REMARKS

I. Status of the Claims

Claims 1-21, 23, and 27-29 are pending. Claims 19-21 are allowed. Claim 1 has been amended to replace a period (".") with a semicolon (";") before the last paragraph. Claims 16-18 and 28 has been amended to place them in independent form. Support for this amendment can be found in original claim 1 and the specification. Claim 26 has been cancelled without prejudice or disclaimer. Claim 27 has been amended to be directed to asthma and atopic dermatitis. New claim 30 has been added, support for claim 30 can be found in original claim 27 and the specification. No other claims have been amended in this Response.

In the last Office Action, the Office:

(a) Objected to claims 16-18 and 28 as being dependent upon a rejected base claim;

(b) rejected claims 26 and 27 under 35 U.S.C. § 112, first paragraph;

(c) rejected claims 1-15, 23 and 29, under 35 U.S.C. § 102(b) and § 102(e).

Applicants respectfully traverse the rejections of claims 1-15, 23, 26, 27, and 29, and traverse the objection to claims 16-18 and 28.

II. Claim Objection:

The Office Action objected to claims 16-18 and 28 as being dependent upon a rejected base claim, but stated that claims 16-18 and 28 would be allowable if rewritten in independent form (Office Action at Page 13). Applicants amended claims 16, 17 and 28 to be independent claims. Claim 18 depends from claim 17 only. Thus, the current

ground for objection is now moot and Applicants respectfully request that the objection be withdrawn.

III. Claim Rejection Under 35 U.S.C. § 112: Enablement

The Office rejected claims 26 and 27 for failing to comply with the enablement requirement of 35 U.S.C. § 112. The Office Action indicated that "*the specification, while enabling treatment of asthma and atopic dermatitis with Formula (I) compounds, does not reasonably enable treatment of all pathological conditions/diseases susceptible to amelioration by PDE-4 inhibition with Formula (I) compounds (claim 26) or treatment of asthma, chronic obstructive pulmonary disease, rheumatoid arthritis, atopic dermatitis, psoriasis and irritable bowel disease.*" (Office Action at page 2). Applicants have cancelled claim 26 and have amended claim 27 to be directed to the pathological condition or diseases the Office has indicated are enabled. Thus the current ground for rejection is now moot and Applicants respectfully request that the be withdrawn.

To the extent that some of the subject matter in the previous version of claim 27 is now claimed in claim 30, Applicants respectfully transverse this rejection and request that the rejection be withdrawn. Applicants will address the instant rejection as it applies to new claim 30.

The standard for determining whether the specification meets the enablement requirement is whether "the experimentation needed to practice the invention is undue or unreasonable." M.P.E.P. § 2164.01. *United States v. Teletronics Inc.*, 857 F.2d 778, 8 USPQ 2d 1217 (Fed. Cir. 1988).

Determining whether undue experimentation is required to make and use a claimed invention requires analysis of the *Wands* factors, and such analysis "must consider all the evidence related to each of these factors." M.P.E.P. § 2164.01(a) (citing *In re Wands,* 858 F.2d 731, 740, 8 U.S.P.Q. 2d 1400, 1406 (Fed. Cir. 1988)). The Office Action discussed these *Wands* factors (Office Action at Pages 3-11). Applicants respectfully submit that the Office Action has omitted the analysis of and misinterpreted underlying factual findings in applying the *Wands* factors to the current application.

1. Breadth of the claims:

"As concerns the breadth of a claim relevant to enablement, the only relevant concern should be whether the scope of enablement provided to one skilled in the art by the disclosure is commensurate with the scope of protection sought by the claims." M. P. E. P. § 2164.08. The Office Action stated that the claims cover potentially many compounds of Formula (I) (Office Action at Page 3). Formula (I) is a typical chemical formula representing chemicals such as those disclosed in the specification. Such representative chemical formulas are widely used in patents involving multiple chemical compounds. In any event, the specification teaches one skilled in the art how to synthesize the disclosed compounds, with multiple synthesis schemes and very detailed examples on how to make the compounds represented by Formula (I). There are 239 examples disclosed in the specification. The mere fact that the claimed Formula (I) represent multiple chemical compounds does not support lack of enablement of claim 30.

The Office Action stated that claim 27 is directed to treating some specific diseases, some of which (such as COPD or IBD) include collection of diseases or family of disorders, and treating those diseases are inadequately enabled based on inhibition of PDE-4. (Office Action at Pages 3-5).

The newly added claim 30 covers treatment of other diseases susceptible to amelioration by inhibition of phosphodiesterase 4 such as chronic obstructive pulmonary disease and rheumatoid arthritis. There is a well established cause-effect relationship between inhibition of PDE-4 and treatment of conditions and diseases described in Claim 30.

For example, with respect to COPD, Lipworth, B.J., Lancet 365:167-75 (2005)("Lipworth") describes results from several randomized, placebo-controlled, clinical trials using different PDE4 inhibitors that show the beneficial treatment of COPD. Lipworth at 172. Additionally, MacKenzie, S.J., Alergology International 53:101-110 (2004) ("*MacKenzie*") also explains that "[a]dvanced clinical trials have proved PDE4 inhibitors to have benefit in chronic obstructive pulmonary disease (COPD), asthma, allergic rhinitis and rheumatoid arthritis." *MacKenzie* at 53, bottom of col. 2. Spina, D., *Drugs* 63(23):2575-2594, 2576, col 2 (2003) ("*Spina.*") also discusses the results of clinical trials using PDE4 inhibitors and reports that "significant improvements in lung function and reduction in exacerbation rates was observed in patients with COPD, without any evidence of adverse effects." *Spina* at 2588, col. 2. These results support Applicants claim of treatment of COPD with PDE4 inhibitors.

MacKenzie clearly states that "[a]dvanced clinical trials have proved PDE4 inhibitors to have benefit in chronic obstructive pulmonary disease (COPD), asthma, allergic rhinitis and rheumatoid arthritis." *MacKenzie* at 53, bottom of col. 2.

Additionally, *Lipworth* and *MacKenzie* are only a small subset of the relevant literature in the area of PDE4 inhibition and associated disease treatments. For example, H.J. Dyke and J.G. Montana, Exp. Opin. Invest. Drugs 8:1301-1325 (1999) ("*Dyke &. Montana*"), discloses clinical results where PDE4 inhibitors were used as anti-inflammatory agents in the treatment of a variety of diseases, including the claimed rheumatoid arthritis, atopic dermatitis, psoriasis, and inflammatory bowel disease. Other diseases mentioned in *Dyke &. Montana* where PDE4 inhibitors are effective include allergic rhinitis, multiple sclerosis, and various central nervous system (CNS) disorders (*e.g.*, Parkinson's disease, and learning and memory impairment).

Stawiski *et al.*, J. Invest. Dermatol. 73:261-263(1979) also demonstrated that topical administration of a PDE4 inhibitor in psoriatic patients improves psoriatic lesions. Haninfin *et al.*, J. Invest. Dermatol. 107:51-56 (1996) showed the use of PDE4 inhibitors in patients with atopic dermatitis.

Various clinical studies of PDE4 inhibitors, such as the known PDE4 inhibitor Rolipram, have demonstrated that these inhibitors, in addition to being potent suppressors of TNF-α production, also decreased clinical activity of arthritis in vivo. *See, e.g.*, Ross *et al.*, J. Immunol. 159: 6253-9259 (1997); Sekut *et al.*, Clin. Exp. Immunol. 100:126-132 (1995); and Nyman *et al.*, Clin. Exp. Immunol. 108:415-419 (1997). In addition, the efficacy of Rolipram as a specific PDE4 inhibitor for the prevention and treatment of experimental colitis (part of irritable bowel disease) has also

been reported (see, e.g., Hartman *et al.*, JPET 292:22-30 (2000) and Diaz-Granados *et al.*, Am. J. Pathol156:2169-2177 (2000)).

Moreover, the state of the art, as indicated by US patents filed before the present application was filed, demonstrate that one of ordinary skill in the art would have reasonably expected that PDE4 inhibitors could be used for the treatment of the diseases recited in claim 30. For example, claim 21 of US Patent No. 6,204,275, filed on February 25, 2000, recites (emphasis added):

> 21. A method [of treating or preventing a PDE IV mediated disease or condition, comprising administering to a patient in need thereof a PDE IV inhibitor], wherein the PDE IV mediated disease or condition is selected from the group consisting of: bladder or alimentary smooth muscle spasm; asthma, cystic fibrosis, chronic bronchitis or inflammatory adult respiratory distress syndrome; eosinophilic granuloma, **psoriasis**, or another benign or malignant proliferative skin disease; endotoxic shock, septic shock, **ulcerative colitis, Crohn's disease**, or reperfusion injury of the myocardium or brain; inflammatory arthritis, chronic glomerulonephritis, atopic dermatitis or urticaria; diabetes, alzheimer's disease, allergic rhinitis, allergic conjunctivitis, vernal conjunctivitis, arterial restenosis or atherosclerosis; neurogenic inflammation; **rheumatoid arthritis**, multiple sclerosis, ankylosing spondylitis, transplant rejection or graft versus host disease; bacterial, fungal or viral induced sepsis and septic shock; rheumatoid or osteoarthritis; cancer, tumor growth or metastasis; cachexia; and depression or memory impairment.

Applicants note that ulcerative colitis and Crohn's disease are collectively known as inflammatory bowel disease. *See, e.g., Dyke & Montana* at 1313, col. 2. Claim 19 of US Patent No. 6,162,830, with a § 371 filing date of February 7, 2000, recites (emphasis added):

> 19. [A] method [for treating a pathological condition associated with a function of PDE-IV, comprising administering to a patient in need thereof a PDE IV inhibitor], wherein the pathological condition is selected from asthma, chronic bronchitis, atopic dermatitis, urticaria,

allergic rhinitis, allergic conjunctivitis, vernal conjunctivitis, inflammation of the eye, allergic responses in the eye, eosinophilic granuloma, **psoriasis**, **rheumatoid arthritis**, gouty arthritis and other arthritic conditions, **ulcerative colitis, Crohn's disease**, adult respiratory distress syndrome, diabetes insipidus, keratosis, atopic dermatitis, atopic eczema, cerebral senility, multi-infarct dementia, senile dementia, memory impairment associated with Parkinson's disease, depression, cardiac arrest, stroke, and intermittent claudication.

Claim 15 of US Patent No. 5,972,936, filed on May 20, 1997, recites (emphasis added):

15. [A] method [for the treatment of a pathological condition capable of being modulated by inhibition of phosphodiesterase IV, comprising administering to a patient in need thereof a PDE IV inhibitor], wherein the pathological condition is selected from the group consisting of asthma, chronic bronchitis, chronic obstructive airways disease, atopic dermatitis, urticaria, allergic rhinitis, allergic conjunctivitis, vernal conjunctivitis, inflammation of the eye, allergic responses in the eye, eosinophilic granuloma, **psoriasis**, **rheumatoid arthritis**, gouty arthritis and other arthritic conditions, **ulcerative colitis, Crohn's disease**, adult respiratory distress syndrome, diabetes insipidus, keratosis, atopic eczema, atopic dermatitis, cerebral senility, multi-infarct dementia, senile dementia, memory impairment associated with Parkinson's disease, depression, cardiac arrest, stroke, and intermittent claudication.

Claim 14 of US Patent No. 5,804,588, filed on May 20, 1997, recites (emphasis added):

14. [A] method [for the treatment of a pathological condition capable of being modulated by inhibition of phosphodiesterase IV, comprising administering to a patient in need thereof a PDE IV inhibitor], wherein said pathological condition is selected from the group consisting of asthma, chronic bronchitis, chronic obstructive airways disease, atopic dermatitis, urticaria, allergic rhinitis, allergic conjunctivitis, vernal conjunctivitis, inflammation of the eye, allergic responses in the eye, eosinophilic granuloma, **psoriasis**, **rheumatoid arthritis**, gouty arthritis and other arthritic conditions, **ulcerative colitis, Crohn's disease**, adult respiratory distress syndrome, diabetes insipidus, keratosis, atopic eczema, atopic dermatitis, cerebral senility, multi-infarct dementia, senile dementia,

memory impairment associated with Parkinson's disease, depression, cardiac arrest, stroke, and intermittent claudication.

The Office is invited to review the claims of several other issued patents with similar claims. As can be seen from the foregoing review, the teachings of the specification and the state of the art at the time the application was filed enable one of ordinary skill in the art to practice the subject matter of claim 30.

2. Nature of the invention and predictability in the art:

The Office Action stated there are various types of PDE-4 and pharmacological activity in general is unpredictable. (Office Action, Pages 5-7). However, the relationship between PDE-4 inhibition and treatment or prevention of chronic and acute inflammatory diseases and other pathological conditions, diseases and disorders has been well studied and well established. Research indicates that inhibition of PDE-4 produces airway smooth muscle relaxation, suppresses degranulation of mast cells, basophils and neutrophils along with inhibiting the activation of monocytes and neutrophils. See Torphy, "*Phosphodiesterase Isozymes: Potential Targets for Novel Anti-asthmatic Agents*" in *New Drugs for Asthma*, Barnes, ed. IBC Technical Services Ltd., 1989. Multiple patents have been granted to various PDE-4 inhibitors based on the pharmacological activity of PDE-4 inhibition, such as U.S. 7273875, U.S. 7235579, U.S. 7226930, and U.S. 7087625. Thus, for the area of treatment with PDE-4 inhibitors, the level of predictability is such that the effect of PDE-4 inhibition has been well studied and can be expected from compounds that display PDE-4 inhibition.

In addition, the Office Action overemphasized the alleged unpredictability of the art, without sufficiently considering the other relevant factors such as the amount of direction provided in the specification.

3. Direction and Guidance:

The Office Action stated there is "*no specific direction or guidance provides a regimen or dosage effective specifically for conditions other than asthma and atopic dermatitis.*" (Office Action, Page 7). However, as mentioned above, the nature of PDE-4

inhibition is such that one of ordinary skill in the art would have no problem in determining such data. For example, the dosage of PDE-4 inhibitors is described and discussed in multiple multiple patents and publications describing other PDE-4 inhibitors, as well as methods of treatment that use PDE-4 inhibitors.

4. State of the prior art:

As described above, the relationship between PDE-4 inhibitors and treatment of certain diseases and conditions are well-established. Other PDE-4 inhibitors have been described in granted patents. At the time the application was filed, there was a high level of skill in the art in using PDE-4 inhibitors to treat a pathological condition or disease.

5. and 6. Working Examples and skill of those in the art:

The Office Action stated *"no examples show treatment of any disorder."* (Office Action at Page 7). However, as discussed above, the cause-effect relationship between inhibition of PDE-4 and treatment of the disorders mentioned in claim 30 has been well established. Multiple patents on PDE-4 inhibitor compounds that include method claims for treating PDE-4 related disorders, such as U.S. 7273875, U.S. 7235579, U.S. 7226930, U.S. 7087625, have been granted. These patents demonstrate the existence of the level of skill necessary to enable the claim. The Office Action cited articles saying "*results concerning clinical efficacy of this potent and selective PDE4 inhibitor are anxiously awaited*" and "*the possibility of a combined approach using VIP together with a PDE inhibitor merits further investigation.*" (Office Action at Page 9) These quotations do not deny or undermine the cause-effect relationship between PDE-4 inhibition in

general and treatment of related disorders because they refer to one of many known PDE-4 inhibitors and to a combination therapy respectively.

The Office Action stated "*the FDA has not approved any PDE-4 inhibitor for treatment of any disorder.*" (Office Action at Page 10.) "However, considerations made by the FDA for approving clinical trials are different from those made by the PTO in determining whether a claim is enabled." *See Scott v. Finney*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994). M.P.E.P. 2164.05 (Edition 8, Release 6, September 2007).

The specification discloses multiple working examples to teach how to make the PDE-4 inhibitors claimed. These working examples enable the skilled artisan to practice the method of claim 30 by using the new PDE-4 inhibitors disclosed to treat the pathological condition or disease susceptible to amelioration by inhibition of PDE-4.

7. Quantity of experimentation needed to make or use the invention.

The Office Action stated that "*substantiation of utility and its scope is required when utility is 'speculative', 'sufficiently unusual' or not provided.*" (Office Action at Page 11). However, the utilities of PDE-4 inhibitors are not speculative or sufficiently unusual. As discussed above, there are multiple scientific publications affirming the cause-effect relationship between PDE-4 inhibition and treatment of diseases and conditions, and there there are multiple patents granted to PDE-inhibitors demonstrating pharmacological utility.

Moreover, a disclosure may be enabling even if it requires a considerable amount of complex experimentation, provided the experiments are "merely routine."

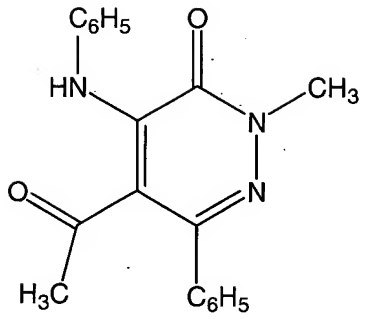
M.P.E.P. § 2164.06. For example, the *Wands* court found an immunoassay method to be enabled, despite the fact that one of ordinary skill in the art would have had to make and screen many antibody-producing hybridoma cell lines to obtain monoclonal antibodies suitable for practicing the invention. *In re Wands,* 858 F.2d at 736. Applying PDE-4 inhibitors disclosed in the specification to treat disorders covered in claim 30 would be routine experimentation to one of ordinary skill in the art, who would have no problem following the well-established cause-effect relationship between PDE-4 inhibition and pharmacological treatment and following the teachings in the specification.

In sum, when presented with the specification's detailed explanation of how to make and use new pyridazin-3(2H)-one derivatives for PDE-4 inhibition, a skilled artisan can practice the invention. For the reasons stated above, claim 30 are enabled. Accordingly, Applicants respectfully request that this rejection be withdrawn.

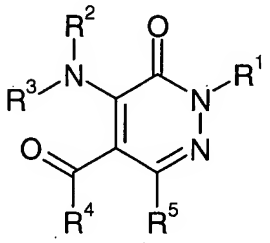
IV. Claim Rejection Under 35 U.S.C. § 102: Anticipation

The Office rejected Claims 1-15, 23 and 29 under 35 USC § 102(b) over Dal Piaz, et al., J. Pharm. Sci. Vol. 80, No.4, April 1991, 341-348 (hereafter "*Dal Piaz*"). The Office Action stated "See Table III, page 344, Compound 4g." (Office Action at Page 12.)

Compound 4g of the *Dal Piaz* reference does not anticipate the instant claims because it is not within the scope of the claims due to the proviso at the end of claim 1. Compound 4g in the *Dal Piaz* reference is 5-Acetyl-2-methyl-4-anilino-6-phenylpyridazin-3(*2H*)-one, with a chemical structure of



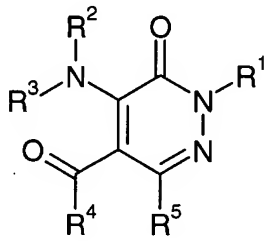
Compare with Formula (I) of claim 1 (claims 2-15, 23, and 29 all depend on claim 1):



(I)

Using the same nomenclature as in the instant claims for compound 4g, R^1 would be methyl, R^5 would be phenyl, R^4 would be methyl, R^2 would be hydrogen and R^3 would be phenyl (or R^2 would be phenyl and R^3 would be hydrogen). Claim 1 states that "when R^5 is neither an optionally substituted heteroaryl group nor a group $COOR^7$, R^3 is an optionally substituted heteroaryl group" (See Claim 1 above.) Compound 4g does not comply with this proviso: in compound 4g, R^5 is phenyl, which is "neither an optionally substituted heteroaryl group nor a group $COOR^7$", but R^3 is either a phenyl or a hydrogen, *not* "an optionally substituted heteroaryl group." Thus, compound 4g is not a species of the genus covered in claim 1, and, therefore, does not anticipate claim 1 (or claims 2-15, 23, and 29, which all depend on claim 1). Accordingly, Applicants respectfully request that this rejection be withdrawn.

The Office also rejected Claims 1-15, 23 and 29 under 35 USC § 102(e) over WO 03/097613 to Dal Piaz, et al. (hereafter "*Dal Piaz II*"). The Office Action stated "See Compounds 1-274 in Table 2, pages 44-78." (Office Action at Page 12.) All those compounds cited can be represented in the same formula (I),



(I)

where "R^3 and R^5 each independently represent a monocyclic or bicyclic aryl group" (Page 4 of the specification in *Dal Piaz II*). This is different from the scope of claim 1, which states "with the proviso that when R^5 is neither an optionally substituted heteroaryl group nor a group $COOR^7$, R^3 is an optionally substituted heteroaryl group." (See Claim 1 above.) In fact, *none* of the compounds listed in Table 2 of *Dal Piaz II* satisfies the proviso of claim 1. For compounds 1-274 in Table 2 of the *Dal Piaz II* reference, R^5 is neither an optionally substituted heteroaryl group nor a group $COOR^7$, *and* R^3 is *not* an optionally substituted heteroaryl group. Thus compounds 1-274 in Table 2 of the *Dal Piaz II* reference cited in the Office Action are not species of the genus covered in claim 1, and do not anticipate claim 1 (or claims 2-15, 23, and 29, which all depend on claim 1). Accordingly, Applicants respectfully request that this rejection be withdrawn.

V. Statement of Common Ownership under 35 U.S.C. § 103(c)

The Office cited WO 03/097613 (*Dal Piaz II*) as a reference qualifying under 35 U.S.C. § 102(e) in an anticipation rejection (Office Action at Page 12).

In an effort to expedite prosecution, and under the provisions of M.P.E.P. §706.02(l)(2), Applicants' undersigned representative of record supplies the following statement to the effect that the instant application and WO 03/097613 were, at the time the invention was made, owned by, or subject to an obligation of assignment to, the same organization, the company now called Laboratorios Almirall S.A.

This application, U.S. Patent Application No. 10/539,821, with an international filing date of December 22, 2003, in the name of Dal PIAZ et al.; and International Patent Application No. PCT/EP03/05056 (WO 03/097613), filed on May 14, 2003, in the name of Dal PIAZ et al.; were, at the time the invention of this application was made, both owned by the same company, or subject to an obligation of assignment to the same company, the company now called Laboratorios Almirall S.A.

As a result, under 35 U.S.C. § 103(c), WO 03/097613 is not applicable prior art under 35 U.S.C. § 103 against the present application.

VI. Conclusion

In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration and reexamination of this application and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

By: _____
Carlos M. Téllez
Reg. No. 48,638

Dated: December 19, 2007

Attachments: Copies of the journal articles cited in this response are enclosed for the Office's convenience

Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease

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Lancet 2005; 365: 167–75

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Inhibitors of phosphodiesterase type 4 (PDE4) act by increasing intracellular concentrations of cyclic AMP, which has a broad range of anti-inflammatory effects on various key effector cells involved in asthma and chronic obstructive pulmonary disease (COPD). The therapeutic ratio for PDE4 inhibitors is thought to be determined by selectivity on receptor subtypes for relative effects on PDE4B (anti-inflammatory) and PDE4D (emesis). The two main orally active PDE4 inhibitors in the late phase III of clinical development are cilomilast and roflumilast; the latter (and its active metabolite N-oxide) is more selective and potent with a superior therapeutic ratio. Studies on cilomilast in COPD based on bronchial biopsy material have shown a broad range of anti-inflammatory activity, and the available evidence on clinical outcomes for up to 6 months with cilomilast 15 mg twice daily and roflumilast 500 μg once daily have shown variable but significant effects on exacerbations and quality of life, with small improvements in measures of pulmonary function. Roflumilast has a better safety and tolerability profile than cilomilast, with the main adverse effects being nausea, diarrhoea, and abdominal pain. Roflumilast also has activity in asthma as assessed by its attenuation of allergen and exercise challenges, and it shows clinical efficacy equivalent to that of beclomethasone dipropionate 400 μg daily. The emerging results of clinical trials on PDE4 inhibitors in asthma and COPD should be interpreted with cautious optimism since much of the evidence has been published only in abstract form to date. The next few years should resolve important issues about the potential role of these drugs as oral non-steroidal anti-inflammatory therapy for asthma and COPD and their place in management guidelines. Ultimately, clinicians will want to know whether PDE4 inhibitors are anything more than expensive "designer" theophylline, the archetypal non-selective phosphodiesterase inhibitor.

Current management guidelines for asthma and chronic obstructive pulmonary disease (COPD) have similarities in terms of the available therapeutic options—namely the use of inhaled corticosteroids along with β_2 agonists, anticholinergic drugs, or theophyllines.[1,2] In general terms, the chronic inflammatory process in asthma is predominantly eosinophilic and in most cases responsive to inhaled corticosteroids, whereas in COPD the inflammation is predominantly neutrophilic and generally refractory to inhaled corticosteroids.[1,2]

There are, however, some unmet needs in current pharmacotherapy of both disorders. In asthma, although the efficacy of inhaled corticosteroids is well established, concerns remain about dose-related local and systemic adverse effects. There is a need for an agent with a wide therapeutic ratio and a broad range of non-steroidal anti-inflammatory activity, to be used either as monotherapy instead of inhaled corticosteroids in milder asthma or as adjuvant therapy to inhaled corticosteroids in more severe asthma—ie, as corticosteroid-sparing therapy. In COPD, there is an even greater need for a non-steroidal anti-inflammatory agent, because inhaled corticosteroids tend to be much less effective in this disorder than in asthma.[3–5] Many prescribers believe that the use of the oral rather than the inhaled route of administration might facilitate better adherence to treatment by patients in the longer term, although this notion remains to be proven. In some respects, for the treatment of asthma, the leukotriene-receptor antagonist montelukast already fulfils the role of once-daily orally active non-steroidal anti-inflammatory therapy, although the available evidence suggests that this drug is less effective than a low dose of inhaled corticosteroid for treatment of mild to moderate persistent asthma.[6–8] A drug with greater anti-asthmatic potency than leukotriene-receptor antagonists would therefore be extremely useful.

Theophylline is also perceived to be an orally active anti-inflammatory agent for use in asthma or COPD. It is the archetypal non-selective phosphodiesterase inhibitor, although it has a narrow therapeutic ratio and several potential important interactions with other drugs, which limit its use for these disorders. A detailed

Search strategy and selection criteria

The search methods for this article included use of PubMed and EMBASE from 1990 to the present with no restrictions on language, with the keywords "phosphodiesterase type 4 inhibitors", "roflumilast", and "cilomilast", as well as searches of relevant abstract books from conference proceedings such as: European Respiratory Society; American Thoracic Society; American Academy of Allergy, Asthma and Immunology; American College of Allergy, Asthma and Immunology; and European Academy of Allergology, Asthma and Immunology. Abstracts lack the detail and authority of fully peer-reviewed papers, but those referred to are cited critically and abstracts are used only when no paper has yet been published or is in press. In many instances, however, details were available from the posters that were presented at the various meetings. I also searched the websites for relevant pharmaceutical companies in addition to contacting them personally for up-to-date information.

review of the anti-inflammatory properties of theophylline is given elsewhere.[9]

Inhibitors of phosphodiesterase type 4

The breakdown of the cyclic nucleotides cAMP and cGMP to their respective 5'-nucleotide monophosphates is catalysed by phosphodiesterase enzymes. 11 families of phosphodiesterases have been categorised so far, differing in their sequence, substrate specificity, cofactor requirements, and sensitivity to inhibitors.[10] This review focuses on the inhibitors of type-4 phosphodiesterase (PDE4), a group of pharmacologically distinct enzymes encoded by at least four distinct genes (*PDEA, PDEB, PDEC, PDED*), which have specificity for cAMP.[11]

PDE4 is ubiquitous among inflammatory and immune cells. PDE4 inhibitors show suppressive activity on various in-vitro responses, including production of cytokines, cell proliferation and chemotaxis, release of inflammatory mediators, and NADPH oxidase activity.[12] Moreover, PDE4 inhibitors have evident activity in vivo in animal models of airway inflammation.[13] cAMP acts via a cascade of protein kinases; the resultant catalytic domains are free to translocate within the cell and phosphorylate specific targets, including transcription regulators, ion channels, and signalling proteins. These events in turn result in actions on various inflammatory and accessory cells, including epithelial cells, dendritic cells, eosinophils, macrophages, mast cells, monocytes, basophils, neutrophils, and T and B lymphocytes.

Pharmacology

Understanding of some of the basic pharmacology of PDE4 inhibitors is important for appreciation of how their therapeutic ratio can be refined and the pharmacologically predictable adverse effects of the class as a whole can be avoided. Initial attention focused on the possible role of the rolipram binding site. PDE4 is thought to have two conformations, $PDE4_H$ and $PDE4_L$,[14] for which the specific inhibitor rolipram has high and low affinity. PDE_H is generally expressed in the central nervous system, producing the adverse effects of nausea and vomiting, whereas $PDE4_L$ is associated with anti-inflammatory activity. Thus, although the archetypal PDE4 inhibitor rolipram has much higher potency for $PDE4_H$ than for $PDE4_L$, compounds such as cilomilast and roflumilast both show much lower potency for $PDE4_H$, which results in greater selectivity and a higher therapeutic ratio.[15] However, the high-affinity binding site is now thought not to be relevant because it does not show a close relation with adverse effects when extended series of PDE4 inhibitors have been examined.

Compound (trade name)	Company	Status
Roflumilast (Daxas)	Altana	Phase III: submitted file for EU approval in COPD and asthma
Cilomilast (Ariflo)	GlaxoSmithKline	Phase II: approvable letter from FDA in COPD and possible EU file pending; discontinued for asthma 2003
Arofylline	Almirall Prodesfarma	Phase II/III: detailed status not disclosed
ELB 353	Elbion	Preclinical phase
C-3885	Merck	Phase II: discontinued 2003
V-11294A	Napp	Phase II: discontinued 2003
BAY 19-8004	Bayer	Phase II: discontinued 2001
YM 976	Yamanouchi	Phase I: discontinued 1999

EU=European; FDA=US Food and Drug Administration.

Table 1: PDE4 inhibitors under development or development discontinued

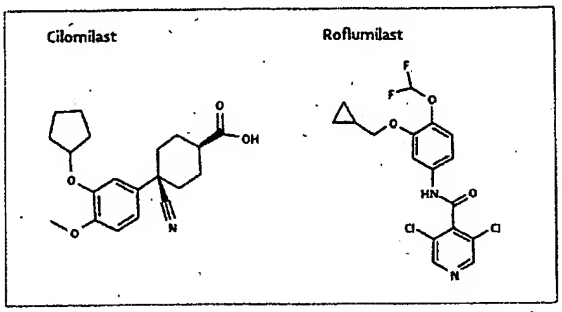

Figure 1: Chemical structures of the two PDE4 inhibitors that are furthest in clinical development

More recent attention has focused on the role of receptor subtypes. PDE4B seems to be the main subtype mediating anti-inflammatory effects, whereas PDE4D mediates the main adverse effect of nausea, through stimulation of neurons in the vomiting centre of the area postrema.[16] Cilomilast is ten times more selective for PDE4D than other isoenzymes, whereas roflumilast and its major metabolite roflumilast N-oxide do not discriminate between PDE4 isoenzymes.[16,17] The selectivity of cilomilast for PDE4D-type nausea-inducing neurons could explain why it appears to be less well tolerated than roflumilast. Whether specific targeting of PDE4B over PDE4D subtypes will achieve a superior therapeutic ratio for future compounds in development remains to be seen.

Several candidate PDE4 inhibitors have been in development, but only cilomilast and roflumilast have proceeded to phase III clinical trials and regulatory submission (table 1, figure 1). The clinical development programme with cilomilast in asthma was stopped by GlaxoSmithKline at phase II, owing to poor efficacy. The drug is currently undergoing late phase III studies in COPD, with an approvable action letter from the US Food and Drug Administration in 2003 for the indication of COPD, and the company has stated that it is still analysing its European Union registration dossier for possible submission. The clinical development programme of roflumilast by Altana is continuing in both asthma and COPD at the stage of phase III trials, and the registration dossier has recently been submitted for European Union registration in 2004 for asthma and COPD. The

remaining part of this review therefore focuses on the two compounds that are nearest to marketing—cilomilast and roflumilast.

Preclinical anti-inflammatory profile of roflumilast and cilomilast

The anti-inflammatory and immunomodulatory activity of PDE4 inhibitors has been investigated in vitro and in animals in vivo. In terms of selective PDE4 inhibition of human neutrophil function, roflumilast was found to be roughly equipotent to its major metabolite, roflumilast N-oxide, and to piclamilast, but it showed potency more than 100 times greater than cilomilast or rolipram (table 2).[17] The rank order of potency for PDE4 inhibitors was similar in human eosinophils. Compared with effects on neutrophils and eosinophils (ie, the terminal inflammatory effector cells), roflumilast and its N-oxide had much greater relative potency than cilomilast for monocytes, CD4-positive T cells, and dendritic cells, suggesting the potential for an improved immunomodulatory profile.[17]

The in-vivo potency of PDE4 inhibitors has also been investigated in classic animal models. For inhibition of the allergen-induced early airway reaction in guineapigs, roflumilast and its N-oxide were equivalent and both were eight times more potent than piclamilast, 32 times more potent than rolipram, and 52 times more potent than cilomilast.[18] Moreover, for the antigen-induced late inflammatory response in sensitised brown Norway rats, orally administered roflumilast and its N-oxide were substantially more potent than the other PDE4 inhibitors for effects on infiltration of eosinophils, neutrophils, and lymphocytes, protein accumulation, and release of tumour necrosis factor α (TNFα; table 3). Orally administered roflumilast and its N-oxide inhibited lipopolysaccharide-induced release of TNFα; they were eight times more potent than piclamilast, 25 times more potent than rolipram, and 310 times more potent than cilomilast. In another in-vivo study of antigen-sensitised Norway brown rats, previous treatment with oral roflumilast compared with placebo significantly delayed (peak at 72 h *vs* 48 h) and reduced (66% at 48 h) eosinophilic infiltration, and significantly inhibited peak expression of mRNA for interleukins 4 and 5 and release of interleukin 13 (81%, 96%, and 77% of respective placebo values), as assessed from bronchoalveolar lavage after allergen challenge.[19] Recent in-vitro studies on guineapig isolated bronchus tested with electrical-field stimulation have shown that roflumilast, and its N-oxide, can regulate neuropeptide release from sensory-nerve terminals, which suggests the potential for suppressing neurogenic inflammation in asthma.[20] The range of anti-inflammatory and immunomodulatory activity with roflumilast and cilomilast suggest a potential therapeutic role in asthma and COPD, with roflumilast showing greater potency.

Isoenzyme	Tissue or cells tested	Mean IC_{50}, μmol/L				
		Roflumilast	Roflumilast N-oxide	Cilomilast	Rolipram	Piclamilast
PDE1	Bovine brain	>10	>10	74	>100	>10
PDE2	Rat heart	>10	>10	65	>100	>10
PDE3	Human platelets	>10	>10	>100	>100	>10
PDE4	Human neutrophils	0·0008	0·002	0·12	0·21	0·001
PDE5	Human platelets	8	>10	83	>100	>10

IC_{50}=concentration causing 50% inhibition. Data from Hatzelmann and Schudt,[17] reproduced with permission from the *Journal of Pharmacology and Experimental Therapeutics*.

Table 2: Selectivity of PDE4 inhibitors

Clinical trials with cilomilast and roflumilast

Pharmacokinetics

The pharmacokinetics of cilomilast have been extensively investigated.[21,22] The drug is rapidly absorbed after oral administration with a t_{max} of about 1 h, 96% oral bioavailability, and a plasma elimination half-life of 7 h; it is subject to negligible first-pass hepatic metabolism. Moreover, cilomilast shows dose-related linear pharmacokinetics which are unaffected by age or by food. The metabolism of cilomilast is extensive, with less than 1% of the administered dose appearing as unchanged drug. There is little potential for interactions with other drugs because none of the pathways that metabolise cilomilast involve cytochrome P450 enzymes that are susceptible to competitive inhibition by other drugs (CYP1A2, CYP2D6, CYP3A4). The only P450 enzyme implicated that catalyses hydroxylation has few other inhibitors or substrates (CYP2C8). Thus, at steady state, cilomilast 15 mg twice daily had no significant effects on the pharmacokinetics of warfarin or digoxin.[23,24] Cigarette smoking does not affect the pharmacokinetics of cilomilast, unlike theophylline, for which there is increased clearance in cigarette smokers with COPD.[25]

The pharmacokinetics of roflumilast and its N-oxide have also been well characterised: they have t_{max} of 1·5 h and 12 h, respectively; elimination half-lives of 10 h and

	ED_{50}, μmol/L				
	Roflumilast	Roflumilast N-oxide	Cilomilast	Rolipram	Piclamilast
Neutrophil	2·2	1·8	90·0	16·0	14·1
Eosinophil	2·7	2·5	106	16·9	23·8
Lymphocyte	2·0	1·6	164	30·6	19·5
TNFα	1·2	1·6	71·2	9·9	14·4
Protein	2·6	1·8	76·9	14·8	19·2

ED_{50}=dose needed to produce specified effect in 50% of animals. Inhibitory effects on differential cell infiltration, protein accumulation, and TNFα release (measured in bronchoalveolar lavage fluid) 48 h after challenge are shown. Data are taken from Bundschuh and colleagues,[18] reproduced with permission from the *Journal of Pharmacology and Experimental Therapeutics*.

Table 3: Inhibitory effects of PDE4 inhibitors on late airway inflammatory response in ovalbumin-challenged brown Norway rats

20 h; with 80% oral bioavailability; no effects of food intake or smoking; diurnal dosing; no interactions with warfarin or erythromycin; and no requirement for dose adjustment in patients with severe renal impairment.[26-30] Moreover roflumilast and its N-oxide do not interact with inhaled salbutamol or inhaled budesonide.[31,32] There are to date no published clinical-trial data on the pharmacokinetics of cilomilast or roflumilast in cor pulmonale or congestive heart failure, which may be relevant because these disorders can reduce the clearance of theophylline and commonly occur in COPD.

Clinical trials in asthma

Inhalation of allergen by atopic asthmatic patients results in immediate bronchoconstriction (early asthma response) caused by release of mediators such as histamine and cysteinyl leukotrienes. This response is followed in half the patients by late bronchoconstriction due to influx of inflammatory cells (late asthma response) and an associated increase in airway hyperresponsiveness. Roflumilast has been investigated in a randomised, double-blind, placebo-controlled study for its effect on the allergen-induced early and late asthmatic responses.[33] 23 patients with mild to moderate asthma (forced expiratory volume in 1 s [FEV_1] 89% of predicted) received once-daily 250 μg roflumilast, once-daily 500 μg roflumilast, or placebo for 7–10 days each, with 2–5-week washout periods between treatments. An allergen challenge was done on the last day of each treatment sequence. Both the early and late asthmatic responses were significantly lower with both doses of roflumilast than with placebo (figure 2). Inhibition of the early asthmatic response amounted to 25% with 250 μg (p=0·008) and 28% for 500 μg (p=0·009) versus placebo. The percentage inhibition of the late asthmatic response was dose related: 27% with 250 μg (p=0·02) and 43% with 500 μg (p=0·0002) versus placebo. In another study with a single 1000 μg dose of roflumilast given 1 h before allergen challenge, there was 62% inhibition of the late asthmatic response (p<0·05).[34] Moreover, roflumilast also attenuated allergen-induced airway hyper-responsiveness to histamine by 1·6 doubling dilutions. These effects of roflumilast on the late asthmatic allergen response are in keeping with the presence of anti-inflammatory activity, as shown in various animal models.

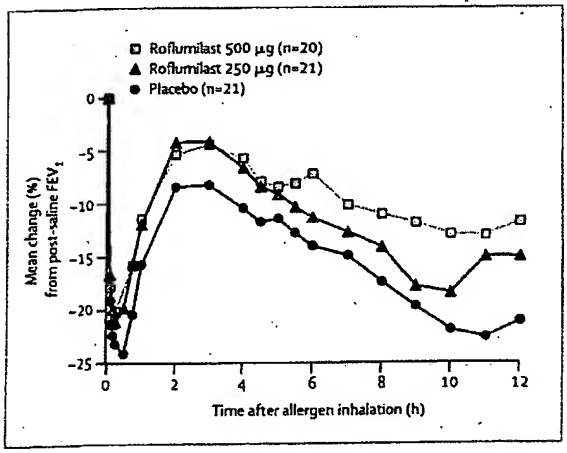

Figure 2: FEV, profile of early and late asthmatic responses to bronchial allergen challenge in patients receiving placebo, roflumilast 250 μg once daily, and roflumilast 500 μg once daily
Data from van Schalkwyk and colleagues.[33]

There is also evidence that roflumilast and cilomilast are effective for protection against exercise challenge. In a study of 16 patients who were randomly assigned placebo or roflumilast 500 μg daily for 4 weeks, the percentage inhibition of FEV_1 fall after exercise was 14% on day 1, 24% on day 14, and 41% on day 28; only the last was significant (p=0·02).[35] Blood samples were taken for measurement of lipopolysaccharide-stimulated TNFα concentrations in whole blood as a marker for inhibition of inflammatory-cell activation. The median TNFα concentration decreased by 21% after roflumilast treatment (p=0·009), but remained constant with placebo (p=0·3). In a study of 27 patients given cilomilast for 1 week at a dose of 10 mg twice daily, there was significant attenuation (p<0·05) of exercise-induced bronchoconstriction, which amounted to 34% inhibition.[36]

In addition to challenge studies, long-term dosing assessments have been made for cilomilast and roflumilast with lung function and other conventional outcome measures of asthma control. In a study of 303 patients in a multicentre, randomised, placebo-controlled, parallel-group trial, cilomilast was given in doses of 5 mg, 10 mg, or 15 mg twice daily for 6 weeks as adjuvant therapy in patients already receiving inhaled corticosteroids. The patients had mean FEV_1 of 66% predicted and at least 12% bronchodilator reversibility to salbutamol.[37] 266 patients completed the study. At the highest dose of cilomilast, there was a greater improvement in FEV_1 than with placebo, but the difference was not significant apart from after 2 weeks when the mean difference between the groups was 0·21 L. Differences between active treatment and placebo in forced mid-expiratory flow and domiciliary peak expiratory flow were not significant. In the patients' overall assessment, 69% of those assigned the highest dose of cilomilast indicated that they were greatly improved compared with 41% of those assigned placebo. For the physicians' overall assessment, 59% of patients assigned cilomilast at the highest dose were improved compared with 29% assigned placebo. In a longer-term (12 months), multicentre, parallel-group trial, cilomilast at a dose of 10 mg or 15 mg twice daily was investigated in 211 patients; the study was an extension of three double-blind, randomised, phase II studies of 4–6 weeks duration.[38] 53 patients had been assigned placebo and 158 cilomilast. There were only small, not significant improvements in FEV_1 over the 12-month period, and

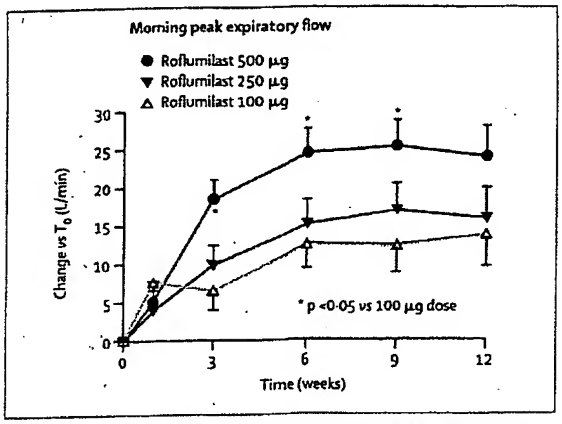

Figure 3: Effects in asthma on FEV_1 and on morning peak expiratory flow from patients' diary cards as change from baseline (mean and SE)
T_0 refers to baseline after placebo run-in period. Data from Leichtl and colleagues.[39]

consequently the further development of cilomilast for the indication of asthma was terminated.

Findings with roflumilast for use in asthma are more promising, as might be predicted from its higher in-vitro potency than cilomilast. In a randomised, double-blind, parallel-group, multicentre, dose-ranging study, roflumilast was given for 12 weeks in doses of 100 μg, 250 μg, or 500 μg once daily with a placebo run-in period of 1–3 weeks before randomisation.[39] At baseline, the mean FEV_1 was 73% of predicted with mean bronchodilator reversibility of 25%; a total of 690 patients were enrolled. Roflumilast led to significant increases in FEV_1 in all three treatment groups ($p<0·0001$), amounting to an 11% increase (260 mL) at 100 μg, 13% (320 mL) at 250 μg, and 16% (400 mL) at 500 μg. The response at 500 μg was significantly better than that to 100 μg ($p=0·0017$; figure 3). Roflumilast also produced significant dose-dependent improvements in morning peak expiratory flow in all three treatment groups ($p<0·006$; 10 L/min, 12 L/min, and 20 L/min, respectively, for 100 μg, 250 μg, and 500 μg doses) as well as significant improvements in evening peak expiratory flow with the 250 μg and 500 μg doses ($p<0·008$). The 500 μg dose was superior to the 100 μg dose ($p<0·05$) for both morning and evening peak expiratory flow (figure 3). In a subsequent 12-month follow-up from the same study, 456 patients received roflumilast 500 μg once daily. Improvements in lung function in patients who had previously received roflumilast 500 μg for 12 weeks were maintained over the 12-month period.[40] In those patients previously treated with the lower doses of roflumilast, there were further increases in FEV_1, which were greatest in those who had previously received the 100 μg dose for 12 weeks ($p<0·006$).

To assess the speed of onset of roflumilast, a pooled analysis was made from three randomised, double-blind, 12-week, multicentre studies, in which 702 patients received roflumilast 500 μg once daily; the mean morning peak expiratory flow was 370 L/min (77% of predicted).[41] The improvement in the peak expiratory flow at day 7 was significant (8 L/min; $p<0·0001$) and it continued through to week 12 (24 L/min; $p<0·0001$). Furthermore, the onset of action was observed early, with significant improvements in morning peak flow (5 L/min; $p=0·006$) noted by day 1; however, a change of this size is unlikely to be clinically relevant.

A comparison of roflumilast 500 μg once daily versus beclomethasone dipropionate 200 μg twice daily over 12 weeks was made in 421 patients with mean FEV_1 of 72% predicted and mean reversibility of 24%, in a double-blind, double-dummy, parallel-group study over 12 weeks with a 1–3-week preceding placebo run-in period.[42] Both treatments produced significant improvements in FEV_1 over the 12-week period (improvement 0·30 L with roflumilast and 0·37 L with beclomethasone; $p<0·0001$; figure 4). Furthermore, both drugs produced significant improvements, of similar size, in morning and evening peak flow ($p<0·001$) and also reduced asthma symptoms and use of rescue medication to a similar degree ($p<0·0001$). The main criticism of all the studies to date with roflumilast in asthma is that they did not include a true randomised placebo group; comparisons were made with baseline values at the end of a 1–3-week non-randomised placebo run-in period. To carry out trials in persistent asthma with a placebo group is becoming more difficult for both ethical and practical reasons, especially because corticosteroid-naive patients are rare in more developed countries, owing to effective implementation of guidelines. Nonetheless, roflumilast was as effective as low-dose inhaled corticosteroid, which would be considered the gold standard for treatment of mild to moderate persistent asthma. For example, in a previous study comparing 400 μg beclomethasone with montelukast 10 mg, the former was significantly superior.[8]

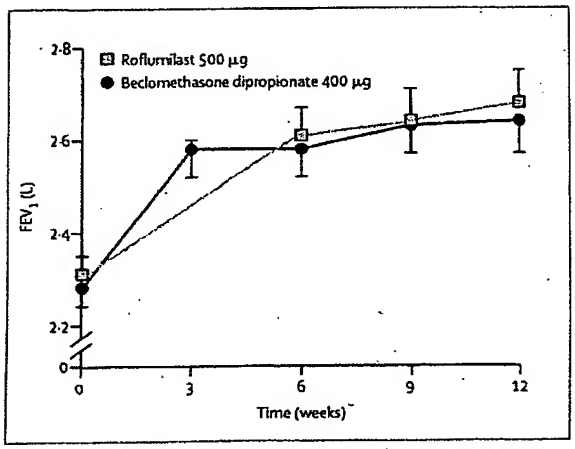

Figure 4: Comparative effects in asthma of roflumilast 500 μg once daily versus beclomethasone dipropionate 200 μg twice daily over 12 weeks on FEV_1 (mean and SE)
Data from Albrecht and colleagues.[42]

In terms of tolerability and adverse events in patients with asthma treated with roflumilast, in the dose-ranging study over 12 weeks (100 μg n=230, 250 μg n=230, 500 μg n=223) the highest frequencies of side-effects were seen with the 500 μg dose for headache (13%), diarrhoea (8%), nausea (8%), and abdominal pain (4%). However, in the long-term follow-up study over a further 40 weeks with the 500 μg dose (n=456) the corresponding rates were 6%, 3%, 1%, and 1%.[39,40] These findings suggest that at least in asthmatic patients there are early dose-limiting adverse events with the 500 μg dose that abate with longer-term treatment. At this point, one cannot say whether the improvement in long-term tolerability with roflumilast at the 500 μg dose is due to patients becoming used to a particular adverse event. In real life, as opposed to the artificial setting of a clinical trial, early adverse effects are more likely to result in patients stopping their treatment. Further long-term, placebo-controlled studies in asthma with the 500 μg dose as well as post-marketing surveillance data will be required to characterise further the real-life adverse-event profile of roflumilast.

Clinical trials in COPD

In a randomised, placebo-controlled, dose-ranging, phase II study of 6 weeks' duration in 424 patients with COPD (FEV_1 46·8% predicted, salbutamol reversibility 5·4%), the doses of cilomilast given were 5 mg, 10 mg, and 15 mg twice daily. The primary outcome was trough FEV_1 before and after bronchodilator use.[41] There was a significant increase in prebronchodilator FEV_1 with cilomilast 15 mg twice daily; the maximum increase compared with placebo was 0·16 L (95% CI 0·09–0·24), occurring at 6 weeks of treatment ($p<0·0001$ *vs* placebo). For postbronchodilator FEV_1, there was also a significant difference from placebo evident at 6 weeks with the 15 mg dose (0·10 L [0·02–0·17]). Significant improvements were also seen in forced vital capacity (mean difference from placebo 0·19 L [0·07–0·31; $p=0·001$) and in peak expiratory flow (difference 34 L/min [18–50]; $p<0·0001$). Quality-of-life measures showed no significant difference between cilomilast and placebo. The most common adverse event was nausea, which occurred in 12% at 10 mg and 11% at 15 mg compared with 1% with placebo. Diarrhoea occurred in 4% with 10 mg, 9% with 15 mg, and 1% with placebo. For adverse events leading to withdrawal of patients, there were 13%, 12%, 11%, and 8%, respectively, in patients taking 15 mg, 10 mg, 5 mg, and placebo, with the most common serious adverse event being an exacerbation of COPD.

In a 6-month, randomised, phase III, double-blind, placebo-controlled, parallel-group, multicentre study, involving 647 patients with COPD (FEV_1 47% predicted, with 7% reversibility), cilomilast 15 mg twice daily significantly improved trough FEV_1 (mean difference 0·08 L; $p<0·001$) and also significantly improved postexercise breathlessness ($p=0·03$) and significantly reduced the risk of exacerbation by 39% compared with placebo ($p=0·002$).[44,45] In addition, there were significant improvements in health-related quality of life; patients achieved a mean reduction in the St George's respiratory questionnaire of 4·1 units ($p<0·001$); a 4-unit change represents clinically meaningful improvement. A pharmacoeconomic analysis from the same study in 340 patients assigned cilomilast and 196 assigned placebo showed that the non-drug cost per patient over the 6-month period was significantly lower for the cilomilast than for the placebo group (incremental non-drug cost saving US$86·29 per patient [95% CI 19·56–187·31]).[46] In terms of tolerability over 6 months with 15 mg twice daily, cilomilast was associated with higher frequencies of diarrhoea (12·7% *vs* 6·0%) and nausea (12·5% *vs* 4·1%) than placebo. These adverse events were generally mild to moderate in intensity and self limiting with or without cessation of treatment.[47]

In a study to investigate the anti-inflammatory profile of cilomilast, a dose of 15 mg twice daily or placebo was given for 12 weeks to 59 patients with COPD; induced sputum testing and bronchial biopsy were done.[48] Cilomilast had no effect on FEV_1 or sputum counts of neutrophils or macrophages compared with placebo and had no effect on supernatant concentrations of neutrophil elastase or interleukin 8. However, on bronchial biopsy, ANOVA showed significant reductions in CD8-positive ($p=0·001$) and CD68-positive cells ($p=0·04$) for cilomilast compared with placebo. Post-hoc Poisson regression analysis confirmed the reductions in the cilomilast group for both CD8-positive (48%, $p=0·004$) and CD68-positive (55%, $p<0·001$) cells. Post-hoc analysis also showed significant reductions in subepithelial CD4-positive cells (42%, $p=0·025$) and neutrophils (37%, $p=0·049$). Although these changes were impressive in terms of biopsy effects in COPD, whether this inflammation relates to features of the disease, such as symptoms, quality of life, or exacerbations, is unclear.

Roflumilast has also been asssessed in a randomised, double-blind, parallel-group, placebo-controlled, multicentre study of 516 patients with COPD (mean FEV_1 54% predicted, 3·6% bronchodilator reversibility); groups were assigned placebo or 250 μg or 500 μg roflumilast once daily over 26 weeks after an initial 2-week run-in period on placebo.[49] The results showed small but significant improvements in FEV_1 ($p<0·0001$) and morning peak expiratory flow ($p<0·012$) with roflumilast compared with placebo. The mean improvements in FEV_1 were 0·057 L for placebo, 0·093 L for 250 μg, and 0·109 L for 500 μg roflumilast; the corresponding improvements in peak flow were 2 L/min, 9 L/min, and 10 L/min (figure 5). The clinical relevance of such small changes in FEV_1 and peak flow is uncertain. In the same study, the numbers of adverse events that were judged to be drug related (eg, nausea, headache, and diarrhoea) were low, and most were mild to moderate in intensity and transient in duration. For the 500 μg dose (n=169) versus placebo

(n=172) over 6 months, the frequencies of headache were 4% versus 2%, abdominal pain 3% versus 1%, nausea 3% versus 2%, and diarrhoea 3% versus 0%. In a 12-month extension study with the 500 μg dose (n=397), the frequencies of the same adverse events were 1% in each case.

There are more recent preliminary data with roflumilast 250 μg or 500 μg once daily over 6 months from a double-blind, placebo-controlled, parallel-group study of 1411 patients with moderate to severe COPD (FEV, 30–80% predicted, <12% reversibility). This study showed small but significant improvements in FEV_1 compared with placebo ($p<0.0001$) with roflumilast 250 μg (0·074 L) and 500 μg (0·097 L), as well as a significant reduction in the mean number of exacerbations per patient ($p=0.0029$) from 1·13 with placebo, to 1·03 with 250 μg and 0·75 with 500 μg (a 34% reduction at the highest dose).[50,51] For health-related quality of life, there was a significant reduction in the St George's score ($p<0.025$), which was greater with 250 μg (–3·25) and 500 μg (–3·51) roflumilast than with placebo (–1·79). The most frequent drug-related adverse events in the roflumilast 250 μg and 500 μg groups were diarrhoea (2% and 6%), nausea (1% and 3%), weight loss (1% and 2%), headache (1% and 2%), and dizziness (0% and 2%). Overall, most drug-related adverse events were mild to moderate in intensity.[51] Moreover, roflumilast did not lead to any clinically significant changes in vital signs, electrocardiographic measures, or laboratory test results. The frequencies of these adverse events therefore seem to be lower in more elderly patients with COPD than in younger patients with asthma.

Although there have been occasional reports from toxicity studies in rats of ischaemic colitis occurring with high doses with certain PDE4 inhibitors in development, there have to date been no published reports of this adverse event with cilomilast or roflumilast at conventional clinical doses. Nonetheless, due vigilance would be needed in patients who developed persistent abdominal pain and diarrhoea, and prompt drug cessation and colonoscopy would be indicated.

The way forward

In patients with mild to moderate persistent asthma at step 2 of the guidelines, further placebo-controlled, multicentre studies will be needed to assess the effects of PDE4 inhibitors on important clinical outcome measures, such as exacerbations, that may be closer to the underlying inflammatory process than measures of airway calibre such as FEV_1 and peak flow. Similar long-term studies powered on exacerbations in mild to moderate persistent asthma would also be indicated for direct comparison of a PDE4 inhibitor with inhaled steroids in low to medium dose (400–800 μg beclomethasone dipropionate or its equivalent) given as monotherapy.

The other issue that will need to be clearly defined for roflumilast and other PDE4 inhibitors in the future is in more severe persistent asthma at step 3 of the guidelines—ie, when used as adjuvant therapy to medium-dose inhaled corticosteroid. Such studies would involve a comparison between addition of a PDE4 inhibitor to patients with uncontrolled asthma on inhaled corticosteroids and use of an increased dose of inhaled corticosteroid, as has been done with other step-3 adjuvant therapies such as long-acting β_2 agonists and leukotriene-receptor antagonists. Subsequently, direct comparison of adjuvant therapy with either PDE4 inhibitor or long-acting β_2 agonist, leukotriene-receptor antagonist, or theophylline would be required.

The evaluation of a PDE4 inhibitor versus other treatments as adjuvant therapy to inhaled corticosteroid at step 3 would require assessment of relative efficacy on surrogate inflammatory markers, such as bronchial biopsy, sputum eosinophilia, airway hyperresponsiveness, and exhaled nitric oxide. At step 4, in patients with severe asthma receiving high-dose inhaled or oral corticosteroid therapy, a back-titration design

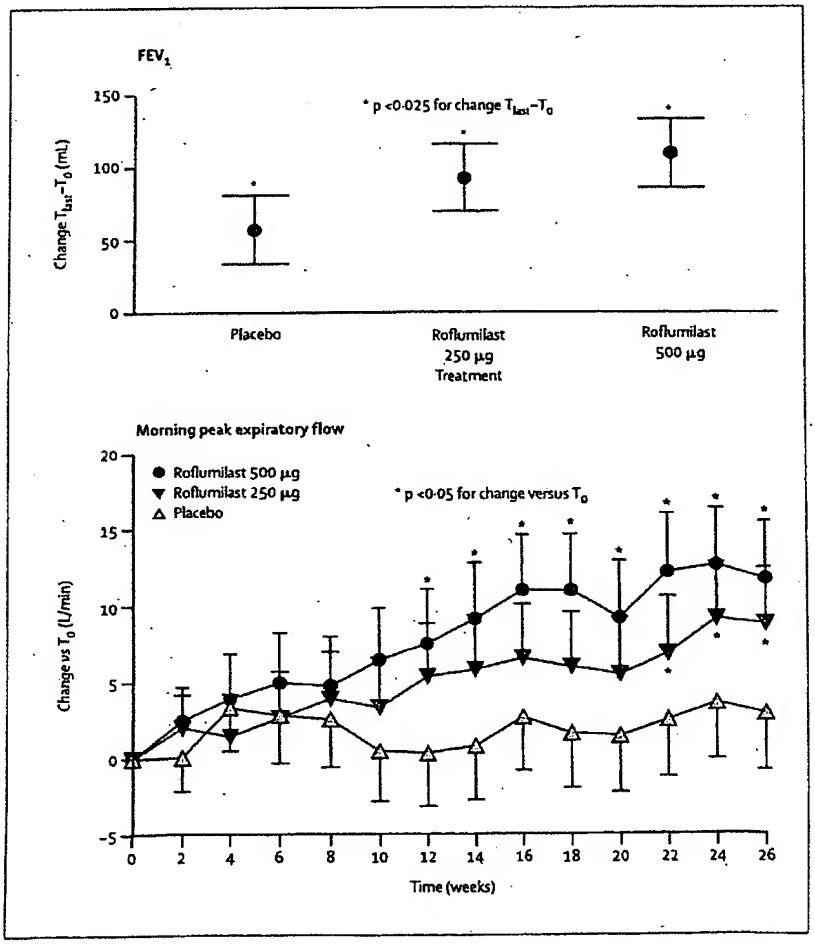

Figure 5: Effects on FEV_1 and morning peak expiratory flow (mean and SE) as change from baseline (T_0) after end of placebo run-in period for 26-week treatment in patients with COPD
Data from Bredenbröker and colleagues.[50]

would be indicated to investigate the putative corticosteroid-sparing effects of an added PDE4 inhibitor.

In COPD, the key study would be a long-term, placebo-controlled assessment over 1 year of a PDE4 inhibitor for its effects on exacerbations and quality of life, and subsequently over 3 years to look also at decline in FEV_1, as has been done with high-dose inhaled corticosteroids. Ultimately, PDE4 inhibitors would have to be compared with other therapies such as long-acting anticholinergic drugs (eg, tiotropium), combination inhalers (eg, fluticasone/salmeterol or budesonide/formoterol), or theophylline, as recommended in current guidelines.

There is also the intriguing possibility in both asthma and COPD that restraining breakdown of cAMP with PDE4 inhibitors might act to potentiate the effect of long-acting β_2 agonists via activation of adenylyl cyclase, which in turn might result in the possibility of synergy, particularly for inflammatory-response outcome measures such as exacerbations. Concerted efforts by the pharmaceutical industry will be needed to address these important issues and to liase with key opinion leaders about the pivotal trials required to implement changes in management guidelines.

In conclusion, therefore, the emerging clinical-trial data on PDE4 inhibitors in asthma and COPD should be interpreted with cautious optimism. The next few years should answer important questions about the potential role of these drugs as oral non-steroidal anti-inflammatory therapy for asthma and COPD. Ultimately, clinicians will want to know whether PDE4 inhibitors are anything more than expensive theophylline.

Conflict of interest statement

The Asthma and Allergy Research Group has received research funding from Altana Pharma, as an unrestricted educational grant, to look at surrogate inflammatory markers in asthma (not with respect to roflumilast). BJL has received occasional personal payments from Altana Pharma for consulting activity (not with respect to roflumilast) and speaking at sponsored postgraduate educational symposia at the European Respiratory Society and the European Academy of Allergology and Clinical Immunology (not with respect to roflumilast). Members of the Asthma and Allergy Research Group (but not BJL) have also received occasional support from GlaxoSmithKline for attendance at postgraduate educational meetings and training. The Asthma and Allergy Research Group have not previously taken part in any clinical trials involving roflumilast or cilomilast. Neither Altana nor GlaxoSmithKline had any involvement with the writing of this report, apart from the supply of up-to-date information on their drugs in development and review of proofs by Altana with the author's and *The Lancet*'s knowledge.

References

1 British Thoracic Society, Scottish Intercollegiate Guidelines Network. British guideline on the management of asthma. *Thorax* 2003; 58 (suppl 1): 1–83.

2 National Collaborating Centre for Chronic Conditions. Chronic obstructive pulmonary disease: National Clinical Guideline on Management of Chronic obstructive disease in adults in primary and secondary care. *Thorax* 2004; 59 (suppl 1): 1–232.

3 Pauwels RA, Lofdahl CG, Laitinen LA, et al. Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pulmonary disease who continue smoking. *N Engl J Med* 1999; 340: 1948–53.

4 Burge PS, Calverley PM, Jones PW, Spencer S, Anderson JA, Maslen TK. Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate to severe chronic obstructive pulmonary disease: the ISOLDE trial. *BMJ* 2000; 320: 1297–303.

5 The Lung Health Study Research Group. Effect of inhaled triamcinolone on the decline in pulmonary function on chronic obstructive pulmonary disease. *N Engl J Med* 2000; 343: 1902–909.

6 Busse W, Raphael GD, Galant S, et al. Low-dose fluticasone propionate compared with montelukast for first-line treatment of persistent asthma: a randomized clinical trial. *J Allergy Clin Immunol* 2001; 107: 461–68.

7 Bleecker ER, Welch MJ, Weinstein SF, et al. Low-dose inhaled fluticasone propionate versus oral zafirlukast in the treatment of persistent asthma. *J Allergy Clin Immunol* 2000; 105: 1123–29.

8 Malmstrom K, Rodriguez-Gomez G, et al. Oral montelukast, inhaled beclomethasone, and placebo for chronic asthma: a randomized, controlled trial. *Ann Intern Med* 1999; 130: 487–95.

9 Barnes PJ. Theophylline: new perspectives for an old drug. *Am J Respir Crit Care Med* 2003; 167: 813–18.

10 Torphy TJ. Phosphodiesterase isozymes: molecular targets for novel antiasthma agents. *Am J Respir Crit Care Med* 1998; 157: 351–70.

11 Spina D. Phosphodiesterase-4 inhibitors in the treatment of inflammatory lung disease. *Drugs* 2003; 63: 2575–94.

12 Souness JE, Aldous D, Sargent C. Immunosuppressive and anti-inflammatory effects of cyclic AMP phosphodiesterase (PDE) type 4 inhibitors. *Immunopharmacology* 2000; 47: 127–62.

13 Essayan DM. Cyclic nucleotide phosphodiesterases. *J Allergy Clin Immunol* 2001; 108: 671–80.

14 Souness JE, Rao S. Proposal for pharmacologically distinct conformers of PDE4 cyclic AMP phosphodiesterases. *Cell Signal* 1997; 9: 227–36.

15 Torphy TJ, Barnette MS, Underwood DC, et al. Ariflo (SB 207499), a second generation phosphodiesterase 4 inhibitor for the treatment of asthma and COPD: from concept to clinic. *Pulm Pharmacol Ther* 1999; 12: 131–35.

16 Manning CD, Burman M, Christensen SB, et al. Suppression of human inflammatory cell functionby subtype selective PDE4 inhibitors correlates with inhibition of PDE4A and PDE4B. *Br J Clin Pharmacol*; 128: 1393–98.

17 Hatzelmann A, Schudt C. Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *J Pharmacol Exp Ther* 2001; 297: 267–79.

18 Bundschuh DS, Eltze M, Barsig J, Wollin L, Hatzelmann A, Beume R. In vivo efficacy in airway disease models of roflumilast, a novel orally active PDE4 inhibitor. *J Pharmacol Exp Ther* 2001; 297: 280–90.

19 Wollin N, Bundschuh DS, Ostermann A, et al. Inhibition of asthma key mediators and inflammation by roflumilast: effects on the time course in ovalbumin challenged brown Norway rats. *Am J Respir Crit Care Med* 2004; 169: A803 (abstr).

20 Boswell-Smith V, Spina D, Page CP. Regulation by selective phosphodiesterase 4 isoenzyme inhibitor of non-adrenergic, non cholinergic contract on guinea pig isolated main bronchus. *Am J Respir Crit Care Med* 2004; 169: A180 (abstr).

21 Zussman BD, Benincosa LJ, Webber DM, et al An overview of the pharmacokinetics of cilomilast (Ariflo), a new, orally active phosphodiesterase 4 inhibitor, in healthy young and elderly volunteers. *J Clin Pharmacol* 2001; 41: 950–58.

22 Zussman BD, Davie CC, Kelly J, et al. Bioavailability of the oral selective phosphodiesterase 4 inhibitor cilomilast. *Pharmacotherapy* 2001; 21: 653–60.

23 Zussman BD, Kelly J, Murdoch RD, Clark DJ, Schübert C, Collie H. Cilomilast: pharmacokinetic and pharmacodynamic interactions with digoxin. *Clin Ther* 2001; 23: 921–31.

24 Kelly J, Murdoch RD, Clark DJ, Webber DM, Fuder H. Warfarin pharmacodynamics unaffected by cilomilast. *Pharmacotherapy* 2001; 35: 1535–39.

25 Kelly J, Murdoch RD, Clark DJ, Zussman B, Davie C, Howland K. Smoking status has no effect on the clearance of a single dose of Arilflo (SP207499)(15mg), an orally active, novel, second generation PDE4 inhibitor in healthy male volunteers. *Am J Respir Crit Care Med* 1999; 159: A807 (abstr).

26 Hauns B, Huennemeyer A, Coch M, Manegold A, Zech K, Bethke TD. Lack of pharmacokinetic interaction between roflumilast and erythromycin. *Am J Respir Crit Care Med* 2004; 169: A612 (abstr).

27 Hauns B, Huennemeyer A, Duursema L, et al. Lack of pharmacokinetic interaction between roflumilast and concomitant warfarin treatment. *Am J Respir Crit Care Med* 2004; 169: A612 (abstr).

28 Manegold A, Hauns B, David M, Zech K, Bethke T, Wurst W. Pharmacokinetic characteristics of roflumilast administered in gradually increasing doses of 500mcg to 1000mcg are dose linear in healthy subjects. *Eur Respir J* 2002; 20 (suppl 38): 108S (abstr).

29 Drollmann A, Hünnemeyer A, Hartmann M, et al. Pharmacokinetic characteristics of roflumilast and its active metabolite roflumilast-N-oxide are not infected by food intake. *Eur Respir J* 2002; 20 (suppl 38): 109S (abstr).

30 Drollmann A, Hartmann M, Zech K, et al. Patients with severe renal impairment do not require dose adjustment of roflumilast. *Eur Respir J* 2002; 20 (suppl 38): 108S (abstr).

31 Weimar C, Bethke T, Westphal K, Zech K, Siegmund W, Wurst W. Roflumilast and its active metabolite, roflumilast-N-oxide do not interact with inhaled salbutamol. *Am J Respir Crit Care Med* 2002; 165: A594 (abstr).

32 Hünnemeyer A, Bethke M, David M, Westphal K, Siegmund W; Wurst W. No interaction of roflumilast and its active metabolite, roflumilast-N-oxide with inhaled budesonide. *Am J Respir Crit Care Med* 2002; 165: A595 (abstr).

33 van Schalkwyk EM, van Heerden K, Bredenbröker D, et al. Dose dependent inhibitory effect of roflumilast, new orally active, selective phosphodiesterase 4 inhibitor on allergen induced early and late asthmatic reaction. *Eur Respir J* 2002; 20 (suppl 38): 110S (abstr).

34 Nell H, Louw C, Leichtl S, Rathgeb F, Neuhäser M, Baden PG. Acute anti-inflammatory effect of the novel phosphodiesterase 4 inhibitor roflumilast on allergen challenge in asthmatics after a single dose. *Am J Respir Crit Care Med* 2000; 161: A200 (abstr).

35 Timmer W, Leclerc V, Birraux G, et al. The new phosphodiesterase 4 inhibitor roflumilast is efficacious in exercise-induced asthma and leads to suppression of LPS-stimulated TNF-alpha ex vivo. *J Clin Pharmacol* 2002; 42: 297–303.

36 Nieman RB, Fisher BD, Amit O, Dochorn RG. SB207499 (Ariflo, a second generation, selective oral phosphodiesterase type 4 (PDE4) inhibitor, attenuates exercise induced bronchoconstriction in patients with asthma. *Am J Respir Crit Care Med* 1998; 157: A413 (abstr).

37 Compton CH, Cedar E, Nieman RB, Amit O, Langley SJ, Sapene M. Ariflo improves pulmonary function in patients with asthma: results of a study in patients taking inhaled corticosteroids. *Am J Respir Crit Care Med* 1999; 159: A522 (abstr).

38 Compton CH, Duggan M, Cedar E, et al. Ariflo efficacy in a 12 month study of patients with asthma. *Am J Respir Crit Care Med* 2000; 161: A505 (abstr).

39 Leichtl S, Schmid-Wirlitsch C, Bredenbröker D, Rathgeb F, Wurst W. Roflumilast, a new orally active, selective phosphodiesterase 4 inhibitor is effective in the treatment of asthma. *Eur Respir J* 2002; 20 (suppl 38): 303S (abstr).

40 Izquierdo JL, Bateman ED. Villasante C, Schmid-Wurlitsch C, Bredenbröker D, Wurst W. Long term efficacy and safety over one year of once daily roflumilast, a new orally active selective phosphodiesterase 4 inhibitor in asthma. *Am J Respir Crit Care Med* 2003; 167: A765 (abstr).

41 Aubier M, Sauer R, Boszormenyi G, Albrecht A, Bredenbröker D, Bethke TD. Roflumilast a novel selective PDE4 inhibitor shows early onset of efficacy in asthma. *Am J Respir Crit Care Med* 2004; 169: A322 (abstr).

42 Albrecht A, Leichtl S, Bredenbröker D, Bethke T, Wurst W. A new orally active, selective phosphodiesterase 4 inhibitor, with beclomethasone propionate in asthma control. *Eur Respir J* 2002; 20 (suppl 38): 304S (abstr).

43 Compton CH, Gubb J, Nieman R, et al. Cilomilast, a selective phosphodiesterase-4 inhibitor for treatment of patients with chronic obstructive pulmonary disease: a randomised, dose-ranging study. *Lancet* 2001; 358: 265–70.

44 Eddleston JD, Compton CH, Neiman R, et al. Cilomilast (Ariflo), a potent selective phosphodiesterase 4 inhibitor reduces exacerbations in COPD patients: results of a six month trial. *Am J Respir Crit Care Med* 2001; 163: A771 (abstr).

45 Eddleston JD, Compton CH, Nieman R, et al. Cilomilast (Ariflo) a potent selective inhibitor of phosphodiesterase 4 improves lung functions in patients with COPD: results of a six month trial. *Am J Respir Crit Care Med* 2001; 163: A277 (abstr).

46 Borker R, Spencer M, Knobil K, Zhu J, Jhingran P. Combining probablistic uncertainty analysis with non-parametric bootstrap procedure to establish the non-drug cost effectiveness of cilomilast in COPD. *Am J Respir Crit Care Med* 2004; 169: A328 (abstr).

47 Compton CH, Eddleston JD, Cedar E, et al. Cilomilast (Ariflo), 15mg bid safety in a 6 month clinical trial programme. *Am J Respir Crit Care Med* 2001; 163: A909 (abstr).

48 Gamble E, Grootendorst DC, Brightling CE, et al. Anti-inflammatory effects of the phosphodiesterase-4 inhibitor cilomilast (Ariflo) in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2003; 168: 976–82.

49 Bredenbröker D, Syed J, Leichtl S, Rathgeb F, Wurst W. Roflumilast, a new orally active, selective phosphodiesterase 4 inhibitor, is effective in the treatment of chronic obstructive pulmonary disease. *Eur Respir J* 2002; 20 (suppl 38): 374S (abstr).

50 Rabe KF, Chapman KR, Jubert J, Vetter N, Witte S, Bredenbroeker D. Roflumilast, a novel selective phosphodiesterase 4 inhibitor improves lung function in patients with moderate to severe COPD. *Am J Respir Crit Care Med* 2004; 169: A518 (abstr).

51 O'Donnell D, Muir JS, Jenkins C, et al. Roflumilast, a novel selective phosphodiesterase 4 inhibitor improves quality of life and lowers exacerbation rates in patients with moderate to severe COPD. *Am J Respir Crit Care Med* 2004; 169: A602 (abstr).

52 Bateman ED, Holmes M, Muir JS, Andrae K, Witte S, Bredenbroeker D. Safety profile of roflumilast, a novel selective phosphodiesterase 4 inhibitor in patients with moderate to severe COPD. *Am J Respir Crit Care Med* 2004; 169: A596 (abstr).

Monthly Focus: Biologicals & Immunologicals

The therapeutic potential of PDE4 inhibitors

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Phosphodiesterase enzymes are responsible for the inactivation of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Phosphodiesterase 4 (PDE4) is a cAMP specific phosphodiesterase expressed in inflammatory cells such as eosinophils. Inhibition of PDE4 results in an elevation of cAMP in these cells, which in turn downregulates the inflammatory response. The anti-inflammatory effects of PDE4 inhibitors have been well documented both *in vitro* and *in vivo* in a variety of animal models. The potential use of PDE4 inhibitors as anti-inflammatory agents for the treatment of asthma and other inflammatory disorders has received considerable attention from the pharmaceutical industry, but to date, there are no selective PDE4 inhibitors on the market. Early PDE4 inhibitors, typified by rolipram, suffered from dose-limiting side effects, including nausea and emesis, which severely restricted their therapeutic utility. Second generation compounds, including CDP840 and SB207499 (Ariflo™), have been identified with reduced side effect liability. Recent evidence suggests a correlation between side effects and the ability of compounds to bind at the so-called high affinity rolipram binding site (HPDE), whilst beneficial effects appear to correlate with binding at the catalytic site. A number of companies are actively pursuing compounds which exhibit improved affinity for the catalytic site and reduced affinity for the HPDE, in the expectation that this will provide compounds with an improved therapeutic index.

Keywords: *allergic rhinitis, anti-inflammatory, Ariflo, asthma, atopic dermatitis, cAMP, chronic obstructive pulmonary disease (COPD), colitis, inflammatory bowel disease (IBD), multiple sclerosis, phosphodiesterase 4 (PDE4), psoriasis, rheumatoid arthritis, rolipram, SB207499*

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Review

1. Introduction

cAMP and cGMP are ubiquitous second messengers that mediate biological responses to a variety of hormones, neurotransmitters, autocoids and drugs [1]. As the concentration of these second messengers rise, they bind to and activate protein kinase A (PKA) and protein kinase G (PKG). These kinases phosphorylate a variety of substrates (such as transcription factors and ion channels) that regulate key cellular responses. Phosphodiesterase (PDE) enzymes are responsible for the hydrolysis of cAMP and cGMP to inactive AMP and GMP, respectively. This results in a corresponding decrease in protein kinase activity and a change of the activation state of a number of biological pathways. Inhibition of PDE enzymes results in an accumulation

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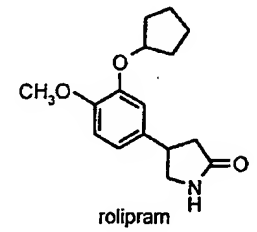
Figure 1: Structures of selective PDE4 inhibitors.



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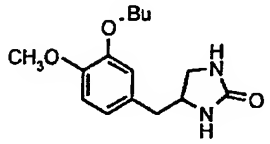


Ro20-1724

of cyclic nucleotides and an increase in protein kinase activity. Thus, the activation state of a number of pathways can be modulated by inhibition of PDE enzymes. The classification of PDE enzymes into seven isozyme families has been described elsewhere [2-4]. Recently, PDE8 [5,6], PDE9 [7] and PDE10 [8] have been characterised. PDE4, first identified in 1985, is specific for cAMP, and is characterised by selective inhibition by rolipram, a synthetic inhibitor described in 1976. Early studies on PDE4 inhibitors were directed at CNS indications, including depression. More recently, the distribution of PDE4 enzyme activity in inflammatory cells has led to increased interest in the use of PDE4 inhibitors as anti-inflammatory agents. The structure, function, regulation and distribution of PDE4 have been reviewed recently [9]. This review will focus on the therapeutic potential of selective PDE4 inhibitors in a variety of diseases, with an emphasis on developments which have occurred in the last three years.

2. Asthma

2.1 Introduction to asthma

Asthma is one of the most common chronic diseases worldwide and constitutes an enormous economic burden to both individuals and society [10]. It is a complex disorder, characterised by intermittent reversible airway obstruction, airway hyperresponsiveness and inflammation [11]. The cause of asthma remains undetermined but the most common pathological expression is now recognised to be inflammation of the airways [11,12]. Considerable inflammation is observed, even in the airways of patients with mild asthma. Bronchial biopsy and lavage studies have demonstrated unequivocal involvement of mast cells, eosinophils and T-lymphocytes as the infiltrating cell types in asthmatic airways [13]. The eosinophils and lymphocytes infiltrating the bronchial wall show markers of activation even in stable asthma [14,15]. Bronchoalveolar lavage in atopic asthmatics shows activation of interleukin (IL)-3, IL-4, IL-5 and granulocyte/macrophage-colony stimulating factor (GM-CSF), suggesting a T-helper 2 (Th2)-like T-cell population [15].

Traditional therapies for asthma fall into four classes [10,15,16], namely bronchodilators (such as β_2-adrenoceptor agonists), anti-inflammatory agents (mainly corticosteroids), prophylactic anti-allergy agents (such as sodium chromoglycate) and xanthines (predominantly theophylline). More recently, modulators of leukotriene activity [17,18] (LTD_4 receptor antagonists, 5-LO inhibitors and FLAP inhibitors) have been approved for the treatment of asthma. β_2 agonists are potent bronchodilators and are useful for the treatment of acute asthma and prevention of exercise-induced asthma, but they do not have anti-inflammatory properties. There is considerable controversy surrounding the regular, long-term use of these agents [15]. Concerns include the development of tolerance, induction of airway hyper-reactivity and a deterioration of asthma control. In contrast, corticosteroids are potent anti-inflammatory agents and reduce the infiltration of mast cells, T-lymphocytes and eosinophils into the bronchial mucosa. Side effects associated with the prolonged use of steroids include dysphonia, oropharyngeal candidiasis, suppression of the hypothalamic-pituitary axis, high bone turnover, reduced bone mineral density and metabolic effects. The significance of many of these effects is disputed [15]. Another limitation of β_2 agonists and steroids is that they are administered as aerosols, which limits patient compliance. Chronic oral administration of these classes of drugs is not currently possible due to side effect liabilities.

Sodium chromoglycate and nedocromil sodium are believed to inhibit mast cell mediator release, including the generation and release of cytokines [13] but their utility as anti-inflammatory agents is disputed [7]. Sodium chromoglycate is only useful in mild to moderate allergic asthma. Nedocromil is more potent, and may allow a reduction in corticosteroid dose. Both of these agents are notable for their lack of

Table 1: *In vivo* effects of selective PDE4 inhibitors.

Inhibitor	Species	Effects observed	References
CP80633 (Atizoram) Pfizer	Monkey and GP	Inhibition of antigen induced EPO in lung, bronchoconstriction, BAL neutrophilia and eosinophilia.	[30]
	GP	Inhibition of histamine-induced bronchoconstriction.	[31]
CP80633	Ferret	Inhibition of antigen-induced pulmonary resistance.	[32]
CP220629 Pfizer	Monkey and GP	Inhibition of antigen-induced bronchoconstriction.	[33]
CP220629	Monkey	Inhibition of antigen-induced lung eosinophilia and neutrophilia.	[33,34]
CDP840 Celltech	GP	Inhibition of antigen-induced bronchoconstriction and leukocyte influx.	[35-37]
CDP840	GP	Inhibition of ozone-induced AHR.	[38,39]
CDP840	GP and rat	Inhibition of antigen-induced lung eosinophilia and IL-5 induced pleural eosinophilia.	[40,41]
CDP840	Rabbit	Inhibition of antigen-induced AHR and eosinophilia, and inhibition of acute bronchospasm.	[42]
CDP840	Monkey	Inhibition of antigen-induced early and late phase bronchoconstriction.	[43]
RP73401 (Picamilast) RPR	GP	Inhibition of ozone-induced AHR.	[38]
RP73401	GP and rat	Inhibition of antigen-induced lung eosinophilia and IL-5 induced pleural eosinophilia.	[40]
RP73401	GP and rat	Inhibition of antigen-induced BAL eosinophilia.	[44]
RP73401	GP	Inhibition of antigen-induced lung eosinophilia and EPO.	[45]
RP73401	GP	Inhibition of antigen-induced airway eosinophilia and PAF-induced AHR.	[46]
SB207499 (Ariflo™) SB	GP	Inhibition of antigen-induced bronchoconstriction and lung eosinophilia.	[47-50]
SB207499	GP	Inhibition of persistent LTD_4-induced lung eosinophilia.	[47]
SB207499	GP	Prevention or reversal of histamine- or LTD_4-induced bronchoconstriction.	[47,48]
T-440 Tanabe Seiyaku	GP	Inhibition of early phase bronchoconstriction induced by antigen, histamine, LTD4, U-46619, ACh, neurokinin A, endothelin-1.	[51,52]
T-440	GP	Inhibition of allergen-induced early phase and late phase bronchoconstriction and BAL eosinophilia.	[53]
T-440	GP	Inhibition of ozone-induced AHR and airway epithelial injury.	[54,55]
T-440	Dog	Inhibition of histamine-induced bronchoconstriction.	[56]
T-440	Rat	Inhibition of antigen-induced bronchoconstriction.	[57]
KF19514 Kyowa Hakko	GP	Inhibition of histamine-induced and antigen-induced bronchoconstriction.	[58,59]
KF19514	GP	Inhibition of PAF-induced lung eosinophilia and antigen-induced BAL eosinophilia.	[59]
KF19514	GP	Inhibition of ACh-induced AHR.	[59]
KF19514	GP	Inhibition of PAF-induced BAL eosinophilia and neutrophilia, and AHR.	[60]

ACh: Acetylcholine; AHR: Airway hyper-responsiveness; BAL: Bronchoalveolar lavage; EPO: Eosinophil peroxidase; GP: Guinea-pig; IL: Interleukin; LTD_4: Leukotriene D_4; PAF: Platelet activating factor.

Table 1: *In vivo* effects of selective PDE4 inhibitors *(continued)*

Inhibitor	Species	Effects observed	References
WAY-PDA-641 (Filaminast) Wyeth-Ayerst Parke-Davis	Dog	Inhibition of seratonin-induced bronchoconstriction.	[61,62]
WAY-127093B Wyeth-Ayerst Parke-Davis	GP	Inhibition of antigen-induced and histamine-induced bronchoconstriction and lung eosinophilia.	[63]
WAY-127093B	Rat	Inhibition of antigen-induced lung eosinophilia and neutrophilia.	[63]
KF17625	GP	Inhibition of antigen-induced bronchospasm.	[64,65]
KF17625	GP	Inhibition of anaphylactic bronchoconstriction.	[66]
V-11294A Napp	GP	Inhibition of antigen-induced bronchoconstriction.	[67]
YM-58997 Yamanouchi	GP	Inhibition of antigen-induced eosinophilia.	[68]
LAS-31025 (Arofylline)	GP	Inhibition of antigen-induced bronchoconstriction.	[69]
CT-2820 Celltech-Merck	Rabbit	Inhibition of antigen-induced bronchoconstriction, AHR and eosinophilia.	[70]
RPR-132294 RPR	Rat	Inhibition of antigen-induced bronchospasm.	[71,72]
RPR-132703	Rat	Inhibition of antigen-induced bronchospasm.	[72]
AWD-12-281 Asta Medica	Rat	Inhibition of allergen-induced late phase eosinophilia.	[73]
AWD-12-281	Rat	Inhibition of early phase mucosal extravasation and late phase eosinophilia.	[74]
AWD-12-281	GP	Inhibition of early phase bronchoconstriction.	[73]
AWD-12-281	Mouse	Inhibition of antigen-induced eosinophilia and AHR.	[75,76]
AWD-12-281	Ferret	Inhibition of LPS-induced neutrophilia.	[77]
PD 168787 Parke-Davis	Rat	Inhibition of antigen-induced eosinophilia.	[78,79]
D-22888 Asta Medica	GP	Inhibition of allergen-induced eosinophilia.	[80]
D-22888	Mouse	Inhibition of allergen-induced AHR.	[81]
D-22888	Pig	Inhibition of antigen induce rhinorrhoea.	[82]
CI-1018 Jouveinal Parke-Davis	Rat	Inhibition of antigen-induced eosinophilia.	[83,84]
UCB29936	Rat	Inhibition of sephadex-induced AHR.	[85]
D4418 Chiroscience Schering-Plough	GP	Inhibition of antigen-induced early and late phase bronchoconstriction and BAL eosinophilia.	[86]

ACh: Acetylcholine; AHR: Airway hyper-responsiveness; BAL: Bronchoalveolar lavage; EPO: Eosinophil peroxidase; GP: Guinea-pig; IL: Interleukin; LTD_4: Leukotriene D_4; PAF: Platelet activating factor.

systemic side effects and provide an alternative therapy for mild asthmatics. Theophylline has a narrow therapeutic index and long-term maintenance therapy with this agent is no longer recommended. Side effects include tachycardia, headache and nausea. The mechanism of action of theophylline has been the subject of intense debate, but it is now generally acknowledged that inhibition of PDE4 accounts for at least some of the beneficial attributes of theophylline [19-22]. Indiscriminate inhibition of all

Table 2: Clinical status of selective PDE4 inhibitors.

Drug	Company	Status	Reference
Arofylline	Almirall	Phase III	[120,121]
Ariflo	SmithKline Beecham	Phase III	[122-128]
Atizoram	Pfizer	Phase II discontinued (under development for atopic dermatitis)	[118]
CDP-840	Celltech	Phase II discontinued	[129-133]
Filaminast	American Home Products	Phase II discontinued	[118]
Piclamilast	Rhône-Poulenc Rorer	Phase II discontinued	[134-136]
V-11294A	Napp	Phase II	[118,137,138]
D4418	Chroscience	Phase I discontinued	[86]
CC-3052	Celgene	Phase I	[118]
CI-1018	Parke-Davis	Phase I	[84]
D-22888	Asta Medica	Phase I	[118]
YM-58997	Yamanouchi	Phase I	[118]
Bay-19-8004	Bayer	Phase I	[139]

PDEs in all tissues of the body by theophylline is responsible for many of the side effects encountered with elevated plasma concentrations of this drug [23,24]. This realisation has prompted the search for a more potent and selective PDE4 inhibitor.

2.2 The role of PDE4 in asthma

PDE4 is specific for cAMP, and is the predominant PDE found in the inflammatory cells associated with asthma. Inhibition of PDE4 results in the elevation of cAMP, which suppresses the activity of immune and inflammatory cells. The distribution of PDE4 in inflammatory cells and airway smooth muscle, and the effects of selective PDE4 inhibitors *in vitro* have been reviewed recently [9]. Effects that have been demonstrated include the inhibition of the release of leukotrienes and histamine from basophils and inhibition of CD11b expression, reactive oxygen species production, chemotaxis and degranulation from eosinophils. In addition, PDE4 inhibitors decrease the release of tumour necrosis factor (TNF)-α from macrophages and monocytes and reduce the production of IL-2, IL-4, IL-5, IL-13 and interferon (IFN)-γ from T-lymphocytes. There is no doubt that selective PDE4 inhibitors exert potent anti-inflammatory effects *in vitro* and thus should have benefits in the treatment of asthma. Bronchodilation with selective PDE4 inhibitors is less convincing, although significant effects can be achieved with mixed PDE4/PDE3 inhibitors or by using a combination of a selective PDE3 and a selective PDE4 inhibitor [25,26].

One of the first selective PDE4 inhibitors to be discovered was rolipram [27,28] (Schering AG) (**Figure 1**), which was initially developed as a potential treatment for depression. This compound has been the subject of numerous *in vivo* studies and has shown efficacy in many animal models, including models of asthma. Another early inhibitor was Ro20-1724 [29] (Roche) (**Figure 1**), which has also been widely studied *in vivo*. **Table 1** provides a summary of the data generated for a collection of more recent selective PDE4 inhibitors in animal models of asthma. The strutures of the compounds discussed in **Table 1** are provided in **Figure 2-4**.

In addition to the compounds listed above, there are several reports describing the *in vivo* activity of a variety of selective PDE4 inhibitors in animal models [31,59,87-91].

It is clear from the results presented in **Table 1** that selective PDE4 inhibitors show good activity in appropriate asthma models, and would thus be expected to have potential in the treatment of this disease. However, most of the inhibitors described to date suffer from side effects, which limit their utility.

2.3 Side effects of PDE4 inhibitors

The side effects most commonly associated with selective PDE4 inhibitors, including the prototypical

Figure 2: Structures of selective PDE4 inhibitors.

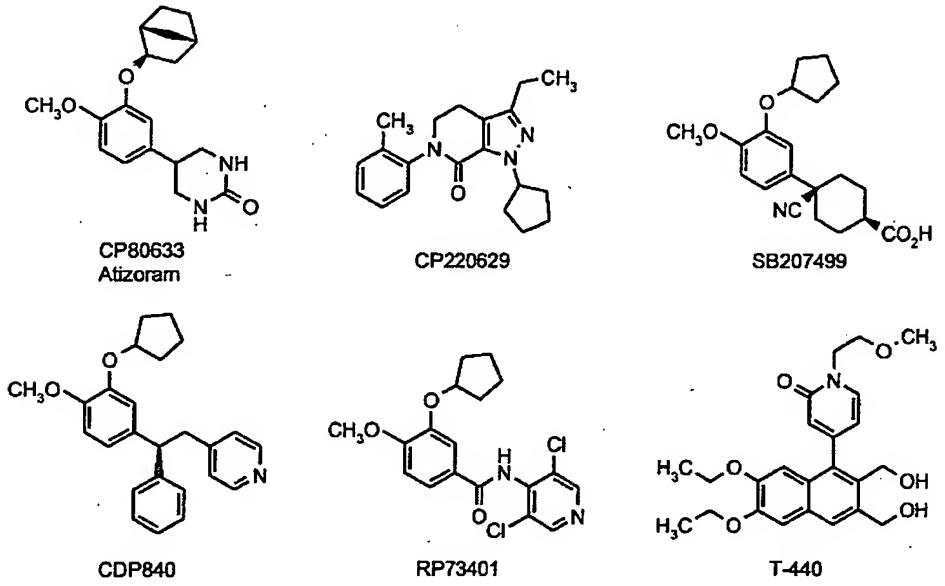


Figure 3: Structures of selective PDE4 inhibitors.

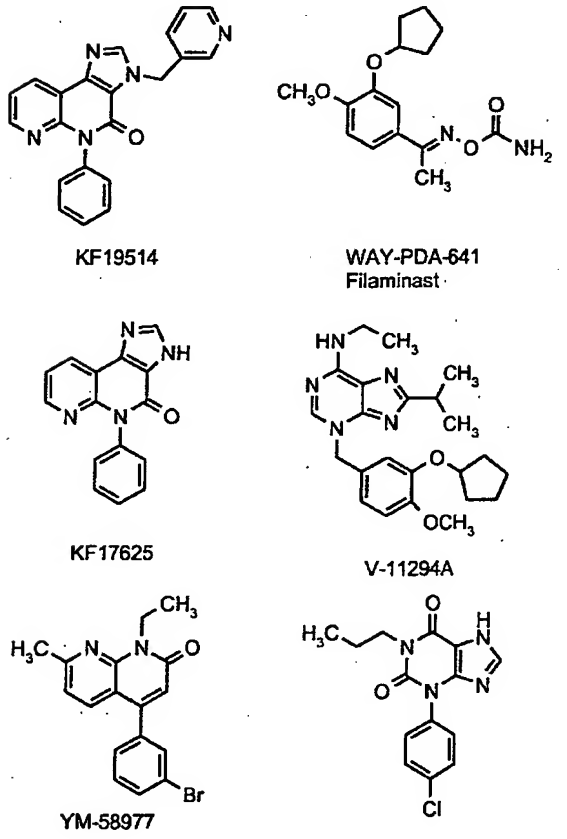
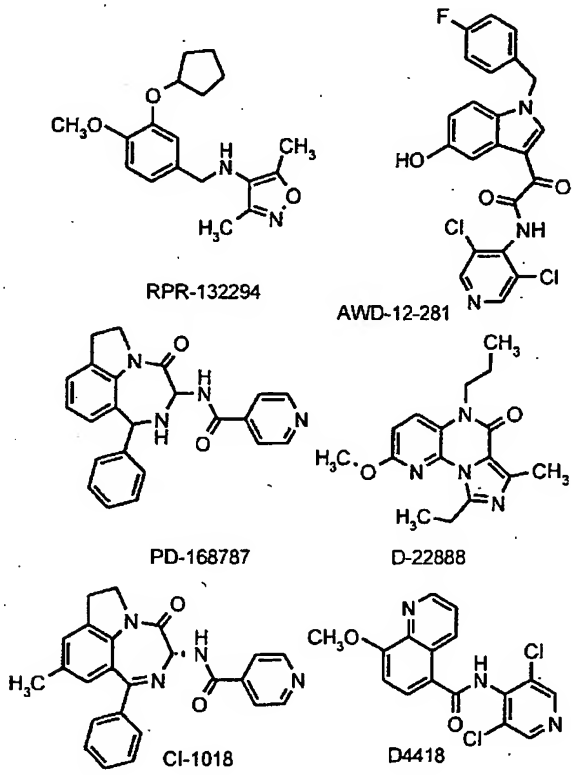


Figure 4: Structures of selective PDE4 inhibitors.



inhibitor rolipram, are nausea and emesis [92-94]. Other reported effects include gastrointestinal discomfort (including increased gastric acid secretion [93,95]) and CNS effects [93,96-100]. In addition to binding at the catalytic site of PDE4 (K_i 0.5 - 1 μM), rolipram has been found to bind with high affinity (K_d ~ 1 nM) at an additional site. This site has been termed the high affinity rolipram binding site, or HPDE4. The nature and function of this site have been the subject of considerable debate, but it is now generally accepted that this site represents an alternative conformation of PDE4 [99-108]. There is evidence to support the theory that the side effects associated with selective PDE4 inhibitors are correlated with the binding affinity of inhibitors with the HPDE4 [9,109-110], whereas therapeutic effects, including anti-inflammatory properties, appear to correlate better with affinity for the catalytic site [9,111-115]. These observation have prompted a number of companies to search for compounds which demonstrate reduced affinity for the HPDE4 and/or improved affinity for the catalytic site. An improvement in selectivity for the catalytic site is expected to translate into an improved therapeutic ratio.

Another approach to reducing side effects has been reported by Pfizer, who have designed inhibitors with reduced CNS penetration [70]. If side effects are centrally mediated, reduced CNS penetration should be beneficial. Synthesis of inhibitors designed to possess reduced ability to enter the brain did not result in reduction in emetic potential. Further structural modifications were designed to reduce high affinity rolipram binding, and compounds with reduced emetic potential were discovered using this latter approach.

The existence of four PDE4 subtypes and differential tissue distribution of these subtypes suggests that subtype selective inhibitors may provide the required separation of therapeutic effects from unwanted side effects. However, there are challenges associated with this approach, including the high sequence homology among the four subtypes. Choosing which subtype to target poses an additional challenge, and it is unclear whether inhibiting a single subtype will have sufficient impact on total cellular cAMP to translate into a biological response. The existence of over twenty PDE4 splice variants is an additional complication which make this approach problematical. The synthesis and biological characterisation of subtype selective inhibitors is required before this approach can be validated [9].

A final strategy, which may allow separation of efficacy from side effects, is to dose directly into the lung thus minimisting systemic exposure to inhibitors [116]. However, there are data which indicate that this approach fails to overcome the side effects of PDE4 inhibitors [117].

2.4 Clinical experience with PDE4 inhibitors

There are currently no selective PDE4 inhibitors on the market. **Table 2** provides a summary of the selective PDE4 inhibitors which have undergone developemnt for asthma. The structures of these compounds are depicted in **Figures 2-5**. The development of drugs of this class has been hampered by adverse events, particularly nausea and emesis. Clinical experience with PDE4 inhibitors has been reviewed recently [118,119].

Arofylline (oral formulation) was found to be effective in a randomised, double-blind, placebo-controlled Phase II study in sixteen patients with mild asthma. A 20 mg dose significantly increased expiratory volume. Lower doses were less effective. No data are available from Phase III studies. An inhaled formulation is currently being evaluated in a Phase I trial.

SB207499 was evaluated in a randomised, placebo-controlled double-blind crossover Phase II trial in twenty seven asthmatic patients. An oral dose of 10 mg was found to protect against exercise-induced asthma and no adverse events were reported with this dose. However, doses of 15 mg and 20 mg were associated with nausea and emesis [123]. In a multi-centre, placebo-controlled, double-blind randomised parallel group Phase III trial in over 300 asthmatics the effect of SB207499 on FEV_1 was measured. The trial studied mild-moderate asthmatics inadequately controlled by inhaled steroids. Significant improvement in FEV_1 with "marked improvement" compared with placebo resulted following b.i.d. administration of 15 mg of SB207499. Lower doses did not produce significant effects, and all doses tested were safe and well-tolerated [124]. Combinations of SB207499 with oral prednisolone [125], theophylline [126] and salbutamol [127] have also been reported.

Clinical evaluation of CDP-840 was discontinued in 1996 due to disappointing efficacy. The development of filaminast has apparently been terminated, and no clinical data have been reported. The development of piclamilast has been discontinued due to problems with side effects and poor pharmacokinetics. Atizoram is under development for atopic dermatitis.

The effects of V-11294A on LPS-stimulated *ex vivo* TNF release and PHA induced lymphocyte proliferation were determined in eight healthy volunteers in a randomised, double-blind placebo-controlled study. An oral dose of 300 mg was found to be effective in reducing TNF levels and lymphocyte proliferation [137,138]. D4418 was administered to healthy volunteers in a single escalating dose, randomised, placebo-controlled Phase I study. No emesis was observed at any of the doses tested, and only mild side effects were noted at the higher doses [86]. CI-1018 was evaluated in 54 subjects and no adverse events were reported at doses up to 400 mg [84]. CC-3052, D-22888, YM-58997 and Bay-29-8004 are thought to be undergoing clinical evaluation, but results have not yet been reported.

3. Allergic rhinitis

Allergic rhinitis is characterised by nasal obstruction, itching, watery rhinorrhea, sneezing and occasional anosmia [140]. It can be divided into perennial and seasonal disease, in which perennial allergic rhinitis can usually be attributed to common allergens, such as house dust mite, animal danders and mould spores, and seasonal disease can be attributed to pollen or outdoor mould spores [141]. Allergic rhinitis is generally made up of an early phase response and a late phase response, reminiscent of asthma. The early phase response is associated with mast cell degranulation [142], while the late phase response is characterised by infiltration of eosinophils, basophils, monocytes and T-lymphocytes. A variety of inflammatory mediators are released by these cells, all of which may contribute to the late phase inflammation [143,144]. The similarities between the pathology of allergic rhinitis and asthma suggest that PDE4 inhibitors should have utility in both diseases. D-22888 has been shown to exert a strong anti-allergic effect and inhibit rhinorrhea in the antigen-challenged pig, and this compound is reported to be under preclinical development for the treatment of allergic rhinitis [82]. AWD-12-281 has been shown to be active in a rat model of allergic rhinitis [74].

4. Chronic obstructive pulmonary disease

4.1 Introduction to COPD

Chronic obstructive pulmonary disease (COPD) is characterised by irreversible, progressive airways obstruction and encompasses emphysema and chronic bronchitis [145]. Chronic bronchitis is associated with hyperplasia and hypertrophy of the mucus secreting glands of the submucosa in the large cartilaginous airways. Goblet cell hyperplasia, mucosal and submucosal inflammatory cell infiltration, oedema, fibrosis, mucus plugs and increased smooth muscle are all found in the terminal and respiratory bronchioles. The small airways have been shown to be the major site of airway obstruction. Emphysema is characterised by destruction of the alveolar wall and loss of lung elasticity. There is a well established link between smoking and incidence of COPD, and several other risk factors have been identified, including exposure to coal dust and various genetic factors [146,147]. Clinical presentation of COPD varies from simple chronic bronchitis without disability to a severely disabled state with chronic respiratory failure. The incidence of this disease is increasing; the economic burden is substantial [148,149].

Inflammation of the airways is characteristic of COPD, and bronchoalveolar lavage fluid and sputum from COPD patients contains increased numbers of neutrophils. Some studies have demonstrated a correlation between neutrophilia and degree of airway obstruction. BAL samples and sputum also contain elevated levels of a number of inflammatory mediators, including IL-8, leukotriene B_4 (LTB_4) and TNF-α. Bronchial biopsy has demonstrated an infiltration with T-lymphocytes and macrophages in the surface epithelium and sub-epithelium. Some studies have also reported increased numbers of neutrophils. Increases in airway eosinophil numbers have been reported only in chronic bronchitics with exacerbations. Biopsies have also demonstrated increased levels of TNF-α and IL-4 [145]. It is clear that neutrophils play a key role in COPD, but further research is required to define the roles played by alveolar macrophages, T-lymphocytes and airway epithelial cells.

Several guidelines for the treatment of COPD have been published, and all advocate cessation of smoking. An important role in control of symptoms is played by bronchodilators, but these drugs fail to alter the progression of the disease [150]. Bronchodilators include β-agonists and anticholinergics. The major advance in this area has been the development of longer acting drugs [151]. Theophylline has also been administered to COPD patients, and has been found to reduce neutrophil counts in induced sputum [152].

The inflammation associated with COPD provides a rationale for the use of steroids, but the benefits are unconvincing [151]. Steroids may be beneficial in slowing the progression of COPD, but as discussed previously, there are side effects associated with this class of drug. There is a clearly a need for novel, safe disease modifying therapies. Several new approaches to the treatment of COPD are under investigation, and these have been reviewed recently [151].

4.2 Role of PDE4 in COPD

PDE4 is the predominant PDE in neutrophils and macrophages [87,153-155]. Inhibition of PDE4 in neutrophils results in reduced chemotaxis, activation, adherence and degranulation [154,156,157]. In peripheral blood neutrophils, PDE4 inhibitors reduce superoxide anion production in response to a number of stimuli [153,154,156,158-160]. Leukotriene synthesis is regulated by PDE4 inhibitors [154], and inhibition of CD11b/CD18 expression is also observed [161,162]. Inhibition of alveolar macrophage PDE4 reduces the release of chemotactic factors and TNF-α [163] and increases IL-10 synthesis [164]. The role of PDE4 in immune and inflammatory cells has been reviewed recently [9].

Whereas asthma has attracted a considerable amount of work, and the effects of a large number of PDE4 inhibitors have been evaluated in animal models, considerably fewer studies have been reported on the effects of inhibitors in models of COPD. Models of COPD are characterised by airway neutrophilia, rather than the eosinophilia measured in asthma models. In a sensitised mouse model, oral administration of T-440 significantly inhibited airway obstruction and neutrophilia induced by antigen provocation [165]. In guinea-pigs, SB207499 inhibited LPS-induced neutrophilia and oedema [166].

4.3 Clinical experience

SB207499 is the only selective PDE4 inhibitor which has been evaluated in COPD patients. In a Phase II trial, treatment with SB207499 15 mg twice a day for six weeks resulted in increases in FEV_1 and forced vital capacity (FVC) [128,167]. Efficacy was also demonstrated in a four week trial, with improvement in FEV_1 and other parameters [128]. A six week Phase III study in COPD patients receiving 15 mg twice a day demonstrated improvement in FEV_1 [128].

5. Rheumatoid arthritis

5.1 Introduction to RA

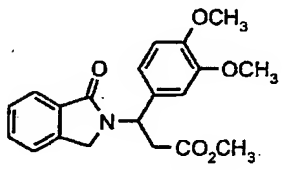
Rheumatoid arthritis (RA) is a crippling autoimmune disease, which affects over 1% of the population. It is a chronic systemic disease of unknown aetiology that produces significant morbidity and some mortality [168]. Three major pathological features contribute to the progressive joint destruction: inflammation; abnormal cellular and humoral responses; synovial hyperplasia [169,170]. The current treatment of RA consists of a combination of non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying antirheumatic drugs (DMARDs). The NSAIDs provide symptomatic relief, but do not have any effect on the underlying disease. These agents also suffer commonly from GI side effects that often limit patient compliance. The DMARDs, such as methotrexate, sulfasalzine and gold salts [171], are very slow acting and have limited patient efficacy [172] with a significant incidence of adverse reactions [173].

The cellular pathology of RA is very complex, but the presence of T-cells and monocytes, and their role in the initiation and development of arthritic lesions is well characterised. T-cells constitute up to 50% of cells recovered from the synovial tissue of RA patients. These cells show an enhanced ability to cross membranes and are found to be predominantly memory T-cells. Of the monocytes found in RA synovium, 30 - 50% are antigen presenting cells, illustrating the high autoimmune component to the disease [174]. There is also considerable evidence now that the pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are the major contributors to joint tissue damage, inflammation, hyperplasia, pannus formation and bone resorption [175]. Indeed, the use of monoclonal antibodies to TNF-α has shown significant promise in RA clinical trials [176].

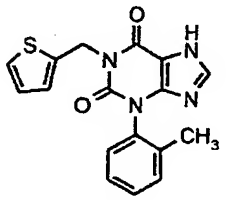
5.2 Role of PDE4 inhibitors in RA

PDE4 inhibitors suppress the action of a wide range of inflammatory cells, including basophils, eosinophils and mast cells [177]. They also possess a very broad *in vitro* anti-inflammatory action *via* inhibition of the release of reactive oxygen species [178], prostaglandins, and inflammatory cytokines such as IL-5 [86,179], IFN-γ [179] and TNF-α [86,178,179]. PDE4 inhibitors also have the potential to address the T-cell component of RA, since they are very effective in inhibiting T-cell proliferation mediated *via* a range of

Figure 5: Structures of selective PDE4 inhibitors.



CC-3052



xanthine 1

different agents including antigens such as house dust mite [179]. They are also capable of facilitating the release of the anti-inflammatory cytokine, IL-10, from monocytes [180]. IL-10 in turn is capable of decreasing the generation of TNF-α, IL-1β and GM-CSF by synovial fluid mononuclear cells [181], thus augmenting the overall anti-inflammatory profile of PDE4 inhibitors.

Of particular interest to the treatment of RA is the potent inhibition of TNF-α released from stimulated monocytes [86,178,179]. This *in vitro* effect translates into animal models of inflammation in which anti-inflammatory effects correlate with suppression of TNF-α accumulation. These include the inhibition of LPS induced TNF-α release in mice by oral administration of CP80633 [182], and the inhibition of rat paw oedema, induced by carageenan, by oral administration of rolipram [183].

The impressive *in vitro* anti-inflammatory profile of PDE4 inhibitors and their utility in the modulation of TNF-α *in vivo* prompted a number of groups to explore the potential of PDE4 inhibitors in animal models of RA. An early disclosure from Glaxo linked the anti-inflammatory activity of the archetypal, selective PDE4 inhibitor, rolipram, in acute inflammation models with significant effects in a mouse adjuvant arthritis model [184]. More recently, the same compound has been shown to reduce disease severity in collagen II induced arthritis (CIA), in the mouse after sc. [185] or ip. injection [186].

A considerable amount of work has been reported in this area from workers at Rhone-Poulenc Rorer (RPR). They have investigated the effect of selective PDE4 inhibitors in both CIA and streptococcal cell wall-(SCW) induced arthritis [187]. A range of compounds has been evaluated in these models and they generally show significant amelioration of disease [187-189]. For example, RP73401 exhibited significant improvements in joint destruction, synovitis and fibrosis over the oral dose range 3 - 30 mg/kg [187]. Of particular interest to RA was the finding that RP73401 and related compounds decrease TNF-α mRNA expression at the pannus/cartilage interface of paw joints of collagen II treated mice, again illustrating the anti-TNF activity of PDE4 inhibitors at the site of action of the disease [187]. However, as with the treatment of asthma, the efficacy of PDE4 inhibitors has to be compared with the side effect profile of this class of compound (see Section 2.3). Comparisons of efficacy using a model of TNF-α release in the mouse with emesis in the dog have been undertaken [188]. These studies illustrate that emesis in the dog generally occurs at lower doses than is required for inhibition of TNF-α release. However, the key question with compounds of this type is how does such a therapeutic index in animal studies translate to man?

5.3 Clinical experience

The first selective PDE4 inhibitor to be studied clinically in RA patients was the RPR compound, RP73401. In a small placebo-controlled, double-blind Phase II study of 35 RA patients, RP73401 (400 μg t.i.d.) was able to induce a positive trend towards clinical improvement associated with a reduction in C-reactive protein (CRP) and IL-6 serum levels, with no serious or unexpected adverse events [190,191]. The results from this study were not statistically significant, but this was probably due to the size of the study and its short duration. Earlier Phase I tolerance studies assessed the ability of the compound to suppress *ex vivo* LPS induced TNF-α release in whole blood from healthy volunteers. Suppression of the response was observed at a minimum oral dose of 1.6 mg t.i.d. However, at this dose, the side effects typical of a PDE4 inhibitor, including nausea, were observed. Therefore, it was not possible to determine whether there was a good correlation in man between inhibition of TNF-α release and amelioration of disease [187]. In order for this to be investigated further, PDE4

inhibitors with an improved therapeutic index are required.

6. Atopic dermatitis

6.1 Introduction to AD

Atopic dermatitis (AD) is a chronic inflammatory skin disease which affects some 10 - 15% of the population during childhood. The prevalence of the disease is increasing and there are few, if any, current therapies which are directed at the underlying disease [192]. AD is part of the so-called 'atopic triad' and occurs frequently in individuals with asthma and/or allergic rhinitis [193,194].

Th2 type cytokine secreting T-cells expressing the cutaneous lymphocyte associated antigen play a central role in the induction of local IgE responses and recruitment of eosinophils in this disease. Chronic inflammation in AD probably involves a number of interdependent factors, such as repeated or persistent allergen exposure, which can lead to Th2 cell expansion [195]. A number of reports have demonstrated an increased frequency of allergen specific T-cells producing increased IL-4, IL-5 [196] and IL-13 levels in the blood of AD patients [197]. These are important findings because IL-4 and IL-13 induce the expression of vascular adhesion molecule-1 (VCAM-1), an adhesion molecule involved in the migration of mononuclear cells and eosinophils into sites of tissue inflammation. Also, IL-5 is a key mediator of eosinophil activation, which is a common feature of atopic disease [196].

It is well recognised that increased cAMP concentrations in lymophocytes and basophils are associated with decreased mediator release from these cells [198]. In addition, it has recently been reported that histamine acting on H2 receptors increased cAMP and inhibited IL-4 production in murine Th2 cells. Therefore, it has been postulated that impaired β-adrenergic responses or enhanced PDE4 activity of leukocyte inflammatory responses are present in atopic diseases such as AD. A diminished cAMP response could result from an enhanced PDE4 activity on either a genetic or acquired basis.

6.2 Role of PDE4 inhibitors in AD

Evidence for the hypothesis of diminished cAMP response in atopic disease has been explored by a number of groups, and several comparisons of different cell types from atopic patients and healthy volunteers have been studied [199-201]. These studies illustrate that the increased cAMP-PDE activity in atopic cells correlates with abnormal inflammatory and immune cell function in AD [201]. It has also been shown that the PDE4 enzyme from atopic leukocytes is more sensitive to PDE4 inhibitors than the PDE4 enzyme from normal leukocytes. In this study there was up to a 14-fold increase in the PDE4 inhibitory concentration when the atopic and normal enzymes were compared [201] (e.g. rolipram: AD IC_{50} = 660 nM; normal IC_{50} = 1270 nM). This has also been translated into an increased sensitivity in the inhibition of proliferation of peripheral blood mononuclear cells from atopic donors on treatment with PDE4 inhibitors. For example, rolipram was more effective at inhibiting PHA stimulated AD PBMC proliferation (IC_{50} = 280 nM) than it was PHA stimulated normal PBMC proliferation (IC_{50} = 2600 nM).

Whilst there are no data illustrating the utility of PDE4 inhibitors in animal models of AD, workers at Chiroscience have shown that a structurally diverse range of selective PDE4 inhibitors [202-204] are effective in reducing skin eosinophilia mediated *via* a range of agents in the guinea-pig. For example, the xanthine 1 (**Figure 5**), shows marked inhibition of skin eosinophilia mediated *via* PAF, arachidonic acid, zymosan activated plasma and protein of cutaneous anaphylaxis, illustrating the potential utility of PDE4 inhibitors in eosinophil driven skin diseases [202].

6.3 Clinical experience

One of the advantages of skin disorders such as AD, from a clinical perspective, is the potential of topical treatment. This route of administration provides a way of limiting systemic exposure and hence unwanted side effects. Pfizer have exploited this opportunity with the selective PDE4 inhibitor, atizoram. The compound was originally developed as an oral treatment for asthma, but a short plasma half-life and induction of emesis in man precluded further development for this indication [118]. Therefore, the compound is being evaluated as a topical treatment for AD. Topical atizoram has been evaluated in 20 patients with AD and has shown beneficial effects. When applied bilaterally over eight days, the compound effectively inhibited all of the inflammation parameters tested [205] and showed both qualitative and quantitative improvements with no adverse effects. This is the first study to suggest that

there may be a role for PDE4 inhibitors in the treatment of AD.

7. Psoriasis

7.1 Introduction to psoriasis

Psoriasis is a common skin disease affecting approximately 2% of the population. Annually 1.5 m patients in the USA are seen by physicians for psoriasis, with associated out-patient healthcare costs estimated to be between $1.6 - 3.2 bn [206]. For those who contract the disease, it is usually recurrent and sometimes very debilitating.

The exact cause of psoriasis is unknown, although it appears to be an autoimmune disease with a likelihood for genetic predisposition [207]. There is a large T-cell infiltration in the affected regions of the skin, with CD4+ lymphocytes in the dermis and CD8+ lymphocytes in the epidermis. These lymphocytes secrete IL-2, IFN-γ and TNF-α which alter keratinocyte proliferation and differentiation [208]. In addition, 5 - 10% of psoriasis patients develop psoriatic arthritis, with symptoms that are very similar to RA. Thus, the potential utility of PDE4 inhibitors in RA and the broad spectrum anti-inflammatory action of such compounds suggests that they have the potential to provide a beneficial treatment for psoriasis. As with AD, psoriasis also provides the opportunity for topical treatments, thus reducing the systemic side effect liability.

7.2 Role of PDE4 inhibitors in psoriasis

Only a limited amount of work has been published on the potential utility of PDE4 inhibitors in the treatment of psoriasis. However, a number of groups have investigated the effect of PDE4 inhibitors on skin. It has been shown that treatment of epidermal basal cells, in primary culture, with the PDE4 inhibitor, Ro20-1724, leads to a three fold increase in cAMP concentrations, illustrating that these cells contain active PDE4 protein [209]. A similar study comparing the effects of Ro20-1724 on psoriatic epidermal slices and keratomed psoriatic epidermal slices showed a very marked elevation of cAMP over controls (1395% increase in cAMP in keratomed psoriatic epidermis) [210], suggesting that PDE4 inhibitors may be potentially beneficial in psoriasis.

With regard to the inflammatory component of the disease, PDE4 inhibitors have been shown to inhibit the inflammatory response of a number of mediators *via* either topical [211] or systemic administration [202-204]. For example, rolipram has been shown to inhibit croton oil-induced ear inflammation in the mouse at topical doses as low as 0.03 mg per ear [11]. Also, a range of selective PDE4 inhibitors from Chiroscience have shown marked reduction in eosinophil accumulation in guinea-pigs after challenge with a range of inflammatory mediators, including PAF, protein of cutaneous anaphylaxis and zymosan activated plasma [202-204].

7.3 Clinical experience

As with AD, one advantage of targeting psoriasis as a clinical end point, is the possibility of topical administration. This is the approach that has been adopted with the only PDE4 inhibitor reported to have been investigated clinically. The selective PDE4 inhibitor, Ro20-1724, was investigated in two double-blind studies comparing its effectiveness to vehicle. In these studies it was shown to improve psoriatic lesions, but it was not as effective as intensive occlusive treatment of psoriatic lesions with 0.025% triamcinolone acetamide. However, Ro20-1724 had no adverse systemic or cutaneous effects, suggesting the therapeutic potential of such compounds in the treatment of psoriasis [212]

8. Multiple sclerosis

8.1 Introduction to MS

Multiple sclerosis (MS) is an autoimmune disease which is characterised by chronic inflammation, demyelination and subsequent gliosis within the central nervous system (CNS) [213,214]. MS encompasses several disease subtypes, including primary progressive MS and relapsing remitting MS [215,216]. The subtypes can be distinguished by disease course, type of inflammation [214] and magnetic resonance imaging (MRI) findings [217]. Disease mechanisms may change during the course of the disease, with inflammation playing a major role early on, and a subsequent change in emphasis to demylination and axonal damage [218].

Inflammatory lesions are localised to the white matter of the CNS. Sclerotic plaques, characterised by demylination, are a hallmark of the disease. Oligodendrocytes undergo necrosis, which leads ultimately to the development of demylination [219]. The process of demylination is associated with an infiltrate

composed mainly of T-cells and macrophages [220]. The infiltrating macrophages and local cells, such as astrocytes, microglia and microvascular brain endothelial cells, express major histocompatibility complex (MHC) class II, which implicates these cells in antigen presentation and an inflammatory response [221]. The autoantigens involved in human MS have not yet been elucidated. The basis for the generation or activation of self-reactive T-cells has not been confirmed, but may involve cross-reactivity between endogenous myelin antigens and exogenous bacterial or viral products [222]. A number of pro-inflammatory cytokines, including TNF-α, TNF-β, IL-1, IL-6 and IFN-γ have been identified in the brain of MS patients and their presence is generally associated with active lesions [223,224]. TNF-α has received particular attention, for several reasons. TNF-α mediates myelin and oligodendrocyte damage *in vitro* [5,122], induces astrocytes to express surface adhesion molecules [226] and is associated with disruption of the blood-brain barrieR2 [27]. Animal models provide additional evidence for a role of TNF-α. In experimental allergic encephalomyelitis (EAE), administration of anti-TNF antibodies or soluble TNF receptors provided a protective effect [228-230]. A direct correlation between the level of TNF-α mRNA and progression of EAE has been reported [231], and an increased concentration of TNF-α in the cerebrospinal fluid of MS patients during the course of the disease has been reported [227]. A transgenic mouse overexpressing TNF-α in the CNS showed signs of spontaneous demyelination [232], but observations with transgenic TNF-α knockout mice suggested a protective role for TNF-α [233].

Disease modifying treatments for MS have been reviewed recently [218]. The most promising therapy currently available is the recently approved interferon-β, which is considered as a milestone in MS therapy. Other drugs which have been investigated in clinical trials for MS include corticosteroids, cyclosporin, tacrolimus and a variety of other immunosuppressive and anti-inflammatory agents [218].

8.2 Role of PDE4 in MS

The role of PDE4 inhibitors in MS has been reviewed recently [234]. As discussed in Section 5.2, PDE4 inhibitors reduce TNF-α production, which suggests they may be beneficial in the treatment of MS since TNF-α has been implicated in this disease. Rolipram has been investigated in several EAE models, with mixed results. Rolipram was reported to reduce disease severity in a Lewis rat EAE model, although the effect was temporary. A second study in rats demonstrated delay in disease onset, but no reduction of severity. Histological analysis indicated reduction of severity of inflammatory cell infiltrate [235,236]. The results show that rolipram does not block the immunological induction pathways leading to EAE, but merely suppresses the inflammatory effects of the disease. In a marmoset EAE model, rolipram suppressed the appearance of clinical signs and abolished abnormalities in MRI imaging. Reduced severity of inflammation was again observed, but the beneficial effects of rolipram were again temporary [237]. A recently reported study of the effects of rolipram in chronic relapsing EAE in SJL mice indicated that rolipram ameliorates clinical signs and pathological changes in this model [238]. Overall, these data suggest that PDE4 inhibitors will be effective anti-inflammatory agents, but not disease modifying agents.

8.3 Clinical experience

Rolipram is reported to be undergoing Phase II trials for the treatment of MS [239]. SH-636 (Schering AG), a PDE4 and TNF inhibitor, is undergoing Phase I trials for MS [240]. No clinical data are available for either of these compounds.

9. Inflammatory bowel disease

9.1 Introduction to IBD

Ulcerative colitis (UC) and Crohn's disease (CD), collectively known as inflammatory bowel disease (IBD), are chronic, spontaneously relapsing disorders of unknown cause. These diseases appear to be immunologically mediated and have genetic and environmental components. Although the cause of the diseases remains obscure, the pathogenesis of chronic intestinal inflammation is becoming clearer, due to improved animal models and important advances in immunological techniques [241,242]. IBD is more common in developed countries and is less pronounced in the third world. While the incidence of UC has remained stable, the incidence of CD has increased markedly [243]. IBD typically affects young people, but is often bimodal with a second episode appearing in later life. Traditional therapy for IBD, although helping to induce and maintain disease remission, does little to alter the underlying

fundamental aspects of the disease. New IBD therapies have not developed significantly over the past 20 years, and current therapies that include 5-aminosalicylic acid [244], corticosteroids [245] and immunomodulators such as azathioprine [246], 6-mercaptopurine [247] and methotrexate [248] suffer from a wide range of side effects. Therefore, there is still a significant need for new disease modifying therapies. TNF-α causes immune cell activation, proliferation and mediator release in IBD [249], and it has been detected in the stools and intestinal mucosa of patients with IBD [250], suggesting that inhibition of this pro-inflammatory cytokine should be beneficial in these diseases. Indeed, early clinical studies in CD with TNF monoclonal antibodies have shown significant promise, further illustrating the benefits of such an approach [241,242].

9.2 Role of PDE4 inhibitors in IBD

Again, very little work has been published on the utility of PDE4 inhibitors in IBD. However, it is widely documented that selective PDE4 inhibitors have a marked effect on the inhibition of TNF-α release from peripheral blood mononuclear cells after stimulation with a wide range of mediators, both *in vitro* [86,178,179] and *in vivo* [184]. Workers at Almirall have taken their most advanced PDE4 inhibitor, arofylline, into models of colitis in the rat and have shown beneficial effects. In a dextran sulphate induced model of colitis in the rat, oral administration of both rolipram and LAS31025 showed comparable effects to prednisolone. In each case, the compounds ameliorated bleeding and inflammatory markers [251]. The same group have also shown that LPS induced erythrocyte extravasation in rats and intestinal hypoperfusion in dogs can be attenuated with the selective PDE4 inhibitors rolipram and denbufylline, illustrating that such compounds provide GI protection under these circumstances [252,253]. However, as yet there have been no reports of the clinical utility of such compounds in man.

10. Other diseases

Rolipram was initially developed as an antidepressant, and is still undergoing Phase III clinical trials for this indication. However, there have not been reports of newer PDE4 inhibitors being evaluated for this indication. There is evidence that PDE4 inhibitors will have beneficial effects in other CNS disorders, including Parkinson's disease [254,255] and learning and memory impairment [256-258]. The use of PDE4 inhibitors to treat tardive dyskinesia [301] and drug dependence [302] has been patented. PDE4 is thought to play a major role in controlling dopamine biosynthesis in mesencephalic neurones [259].

Several reports suggest a role for PDE4 inhibitors in ischaemia-reperfusion injury [260-263]. Acute renal failure may also be amenable to treatment by PDE4 inhibitors [264], and their use for this indication has been patented [303].

There has been a small number of publications suggesting a role for PDE4 inhibitors in the treatment of bone disease [265,266] and again this use of PDE4 inhibitors has been patented [304].

A role for PDE4 inhibitors in the treatment of septic shock [267,268], acute respiratory distress syndrome [268] and allograft rejection [269] has been proposed.

There are a variety of other diseases in which PDE4 inhibitors have been suggested to offer potential benefit, including autoimmune diabetes [270], immunoinflammatory hepatitis [271], retinal autoimmunity [272], chronic lymphocytic leukaemia [273] and HIV [274,275]. In addition, patents have appeared claiming the use of PDE4 inhibitors in the treatment of lupus erythematosus [305], kidney and ureter disease [306], urogenital and gastrointestinal disorders [307] and prostrate diseases [308,309].

11. Expert opinion

The discovery of PDE4 inhibitors with an enhanced therapeutic index has provided substantial improvement over the archetypal inhibitors such as rolipram. This has been established in the main from a greater understanding of how the *in vitro* activity of rolipram translates into efficacy and side effect liability, particularly emesis and CNS effects, *in vivo*. It is obvious from the literature that PDE4 inhibitors exhibit a range of *in vitro* and *in vivo* anti-inflammatory properties that indicate potential in the treatment of a wide range of inflammatory conditions.

To date, the clinical data in most therapeutic areas with compounds of this class is inconclusive. However, in the respiratory area we are seeing for the first time a definite therapeutic benefit and an improved therapeutic index over first generation compounds. The data with SB207499 in COPD in particular illustrates that PDE4 inhibitors have a role to play in this disease alongside the existing therapies of

steroids and β-agonists. Some of the less advanced compounds in the clinic, such as the Chiroscience compound, D4418, also illustrate that further improvements in tolerability at increased plasma exposures is achievable. If such compounds also show the efficacy observed with SB207499, this should provide further improvements in therapeutic index. If the clinical data generated in the respiratory diseases is realised in other inflammatory conditions, particularly those, which have no good disease modifying treatments currently, such as RA and MS, then PDE4 inhibitors could have significant market potential. The use of topical applications in diseases such as AD and psoriasis also provide significant market potential even for those compounds that do not have a particularly good therapeutic index, again illustrating that PDE4 inhibitors could provide a variety of effective therapies across a number of disease states.

Bibliography

Papers of special note have been highlighted as:
- • of interest
- •• of considerable interest

1. KREBS EG, BEAVO JA: **Phosphorylation-dephosphorylation of enzymes.** *Ann. Rev. Biochem.* (1979) 48:923-959.
2. BEAVO JA: **Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms.** *Physiol. Rev.* (1995) 75:725-748.
3. CONTI M, NEMOZ G, SETTE C, VICINI E: **Recent progress in understanding the hormonal regulation of phosphodiesterases.** *Endocr. Rev.* (1995) 16:370-389.
4. MANGANIELLO VC, MURATA T, TAIRA M, BELFRAGE P, DEGERMAN E: **Diversity in cyclic nucleotide isoenzyme families.** *Arch. Biochem. Biophys.* (1995) 322:1-13.
5. FISCHER DA, SMITH JF, PILLAR JS, ST DENIS SH, CHENG JB: **Isolation and characterisation of PDE8A, a novel human cAMP-specific phosphodiesterase.** *Biochem. Biophys. Res. Commun.* (1998) 246:570-577.
6. SODERLING SH, BAYUGA SJ, BEAVO JA: **Cloning and characterisation of a cAMP-specific cyclic nucleotide phosphodiesterase.** *Proc. Natl. Acad. Sci.* (1998) 95:8991-8996.
7. FISCHER DA, SMITH JF, PILLAR JS, ST DENIS SH, CHENG JB: **Isolation and characterisation of PDE9A, a novel human cGMP-specific phosphodiesterase.** *J. Biol. Chem.* (1998) 273(25):15559-15564.
8. FUJISHIGE K, KOTERA J, MICHIBATA H *et al.*: **Cloning and characterisation of a novel human phosphodiesterase that hydrolyses both cAMP and cGMP (PDE10A).** *J. Biol. Chem.* (1999) 274:18438-18445.
9. TORPHY TJ: **Phosphodiesterase isozymes. Molecular targets for novel antiasthma agents.** *Am. J. Respir. Crit. Care Med.* (1998) 157:351-370.
   - •• An excellent review.
10. BARNES PJ: **Molecular mechanisms of antiasthma therapy.** *Ann. Med.* (1995) 27:531-535.
11. DRAZEN JM, ARM, JP, AUSTEN KF: **Sorting out the cytokines of asthma.** *J. Exp. Med.* (1996) 183:1-5.
12. PALMA CARLOS AG, PALMA CARLOS ML, CONCEICAO SM, ALCINDA M: **Cytokines and asthma.** *J. Invest. Allergol. Clin. Immunol.* (1997) 7:270-273.
13. HOLGATE ST: **The inflammatory basis of asthma and its implications for drug treatment.** *Clin. Ex. Allergy* (1996) 26:1-4.
14. AZZAWI M, BRADLEY B, JEFFERY PK *et al.*: **Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma.** *Am. Rev. Respir. Dis.* (1990) 142:1407-1413.
15. JAIN P, GOLISH JA: **Clinical management of asthma in the 1990s.** *Drugs* (1996) 52:1-11.
16. BARNES PJ: **Immunomodulation as asthma therapy: where do we stand?** *Eur. Respir. J.* (1996) 9(Suppl. 22):154s-159s.
17. VIANNA EO, MARTIN RJ: **Recent innovations in asthma therapy.** *Drugs Tod.* (1998) 34(4):341-351.
   - • A useful review.
18. PAUWELS RA, JOOS GF, KIPS JC: **Leukotrienes as therapeutic target in asthma.** *Allergy* (1995) 50:615-622.
19. SPINA D, LANDELLS LJ, PAGE CP: **The role of theophylline and phosphodiesterase4 isozyme inhibitors as anti-inflammatory drugs.** *Clin. Ex. Allergy* (1998) 28(Suppl. 3):24-34.
20. RABE KF, MAGNUSSEN H, DENT G: **Theophylline and selective PDE inhibitors as bronchodilators and smooth muscle relaxants.** *Eur. Respir. J.* (1995) 8:637-642.
21. COWARD WR, SAGARA H, CHURCH MK: **Asthma, adenosine, mast cells and theophylline.** *Clin. Ex. Allergy* (1998) 28(Suppl. 3):42-46.
22. RABE KF, DENT G: **Theophylline and airway inflammation.** *Clin. Ex. Allergy* (1998) 28(Suppl. 3):35-41.
23. CHURCH MK, FEATHERSTONE RL, CUSHLEY MJ, MANN JS, HOLGATE ST: **Relationships between adenosine, cyclic nucleotides and xanthines in asthma.** *J. Allergy Clin. Immunol.* (1986) 78:670-675.
24. HOWELL RE, MUEHSAM WT, KINNIER WJ: **Mechanism for the emetic side effect of xanthine bronchodilators.** *Life Sci.* (1990) 46:563-568.
25. TORPHY TJ, UNDEM BJ, CIESLINSKI LB, LUTTMAN MA, REEVES MK, HAY DWP: **Identification, characterisation and functional role of phosphodiesterase isozymes in human airway smooth muscle.** *J. Pharmacol. Exp. Ther.* (1993) 265:1213-1223.
26. DE BOER J, PHILPOTT AJ, VAN AMSTERDAM RG, SHAHID M, ZAAGSMA J, NICHOLSON CD: **Human broncial cyclic nucleotide phosphodiesterase isoenzymes:**

biochemical and pharmacological analysis using selective inhibitors. *Br. J. Pharmacol.* (1992) **106**:1028-1034.

27. CROSSLAND J: **Rolipram.** *Drugs Future* (1988) **13**(1):38-41.

28. CROSSLAND J: **Rolipram.** *Drugs Future* (1998) **23**(1):108-109.

29. SHEPPARD H, WIGGAN G: **Analogues of 4-(3,4-dimethoxybenzyl)-2-imidazolidinone as potent inhibitors of rat erythrocyte adenosine cyclic 3',5'-phosphate phosphodiesterase.** *Mol. Pharmacol.* (1971) **7**:111-115.

30. TURNER CR, COHAN VL, CHENG JB, SHOWELL HJ, PAZOLES CJ, WATSON JW: **The *in vivo* pharmacology of CP-80,633, a selective inhibitor of phosphodiesterase 4.** *J. Pharmacol. Exp. Ther.* (1996) **278**: 1349-1355.
- Useful reference describing Pfizer's compound.

31. WRIGHT KF, TURNER CR, JAYASINGHE-BECK R, COHEN VL, CHENG JB, WATSON JW: **Differential *in vivo* and *in vitro* bronchorelaxant activities of CP-80633, a selective phosphodiesterase 4 inhibitor.** *Can. J. Physiol. Pharmacol.* (1997) **75**:1001-1008.

32. WATSON JW, BECK R, COHAN VL, SHOWELL HJ, PAZOLES CJ, CHENG JB: **Antianaphylactic and side effect activity of the PDEIV inhibitor CP-80,633 in the ferret.** *J. Allergy Clin. Immunol.* (1995) **95**:353.

33. DUPLANTIER AJ, ANDRESEN CJ, CHENG JB *et al.*: **7-Oxo-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridines as novel inhibitors of human eosinophil phosphodiesterase.** *J. Med. Chem.* (1998) **41**:2268-2277.

34. DUPLANTIER AJ, ANDRESEN CJ, CHENG JB *et al.*: **7-Oxo-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridines as novel inhibitors of phosphodiesterase 4.** *Fifth Chemical Congress of N. America* (1997). Abstract 02/897.

35. HUGHES B, HOLBROOK M, ALLEN R *et al.*: **Suppression of antigen-induced airway responses and bronchial hyperresponsiveness by CDP840, a novel stereoselective inhibitor of phosphodiesterase Type IV.** *Br. J. Pharmacol.* (1995) **116**:(Proc. Suppl.) Abstract 6P.

36. LISLE H, HUGHES B, HOWAT D: **The effect of CDP840 in eosinophil and neutrophil models of inflammation:** *Inflamm. Res.* (1995) **44**(suppl. 3):W02/17.

37. HOLBROOK M, KINGABY R, BLEASE K, HUGHES B, HIGGS G: **Anti-inflammatory effects of CDP840, a novel potent and selective phosphodiesterase (PDE) Type IV inhibitor.** *Inflamm. Res.* (1995) 44(Suppl. 3):W12/12.
- Useful reference describing Celltech's compound.

38. HOLBROOK M, GOZZARD N, JAMES T, HIGGS G, HUGHES B: **Inhibition of bronchospasm and ozone-induced airway hyperresponsiveness in the guinea-pig by CDP840, a novel phosphodiesterase Type 4 inhibitor.** *Br. J. Pharmacol.* (1996) **118**:1192-1200.

39. HOLBROOK M, GOZZARD N, HUGHES B: **CDP840 inhibits ozone (O_3)-induced airway hyperresponsiveness (AHR) to inhaled histamine in anaesthetised guinea-pigs.** *Inflamm. Res.* (1995) 44(Suppl. 3):W12/07.

40. HUGHES B, HOWAT D, LISLE M *et al.*: **The inhibition of antigen-induced eosinophilia and bronchoconstriction by CDP840, a novel stereo-selective inhibitor of phosphodiesterase Type 4.** *Br. J. Pharmacol.* (1996) **118**:1183-1191.

41. LISLE H, HOWAT D: **Investigation of the *in vivo* anti-inflammatory properties of the novel and selective PDE4 inhibitor CDP840.** *Int. Congr. Inflamm. Res. Assoc.* (1996): Abract 109.

42. GOZZARD N, EL-HASHIM A, HERD CM *et al.*: **Effect of the glucocorticosteroid budesonide and a novel phosphodiesterase Type 4 inhibitor CDP840 on antigen-induced airway responses in neonatally immunised rabbits.** *Br. J. Pharmacol.* (1996) **118**:1201-1208.

43. JONES TR, McAULIFFE M, McFARLANE CS, PIECHUTA H, MACDONALD D, RODGER IW: **Effects of a selective phosphodiesterase IV inhibitor (CDP-840) in a leukotriene-dependent non-human primate model of allergic asthma.** *Can. J. Physiol. Pharmacol.* (1998) **76**:21-217.

44. RAEBURN D, UNDERWOOD SL, LEWIS SA *et al.*: **Anti-inflammatory and bronchodilator properties of RP73401, a novel and selective phosphodiesterase Type IV inhibitor.** *Br. J. Pharmacol.* (1994) **113**:1423-1431.
- Useful reference describing RPR's compound.

45. BANNER KH: **The effect of selective phosphodiesterase inhibitors in comparison with other anti-asthma drugs on allergen-induced eosinophilia in guinea-pig airways.** *Pulm. Pharmacol.* (1995) **8**:37-42.
- Good review.

46. KARLSSON J-A, SOUNESS J, WEBBER S, POLLOCK K, RAEBURN D: **Anti-inflammatory effects of the novel phosphodiesterase IV inhibitor RP73401.** *Int. Arch. Allergy Immunol.* (1995) **107**:425-426.

47. UNDERWOOD DC, BOCHNOWICZ S, OSBORN RR *et al.*: **Antiasthmatic activity of the second-generation phosphodiesterase 4 (PDE4) inhibitor SB207499 (Ariflo) in the guinea pig.** *J. Pharmacol. Exp. Ther.* (1998) **287**(3):988-995.
- Useful reference describing SB's compound.

48. UNDERWOOD DC, BOCHNOWICZ S, OSBORN R *et al.*: **Anti-asthmatic potential of the second generation phosphodiesterase 4 inhibitor, SB 207499.** *Eur. Resp. J.* (1997) **10**(Suppl.25):313S.

49. CHRISTENSEN SB, GUIDER A, FORSTER CJ *et al.*: **1,4-Cyclohexanecarboxylates: potent and selective inhibitors of phosphodiesterase 4 for the treatment of asthma.** *J. Med. Chem.* (1998) **41**:821-835.

50. UNDERWOOD DC, OSBORN RR, BOCHNOWICZ S, HAY DWP, TORPHY TJ: **The therapeutic activity of SB 207499 (Ariflo™), a second generation phosphodiesterase 4 (PDE4) inhibitor, is equivalent to that of prednisolone in models of pulmonary inflammation.** *Amer. J. Respir. Crit. Care Med.* (1998) **157**:A827.

51. KAMINUMA O, KIKKAWA H, MATSUBARA S, IKEZAWA K: **Inhibitory effect of a novel phosphodiesterase IV inhibitor, T-440, on antigen- and chemical mediator-**

induced bronchoconstrictions in guinea pigs *in vivo*. *Jpn. J. Pharmacol.* (1996) 72:1-8.

52. **T-440**. *Drugs of the future* (1997) 22:729-732.
- Good review of Tanabe Seiyaku's compound.

53. KAMINUMA O, KIKKAWA H, MATSUBARA S, IKEZAWA K: **Effect of T-440, a novel Type IV phosphodiesterase inhibitor, on allergen-induced immediate and late asthmatic reaction and leukocyte infiltration into the airways of guinea pigs.** *Int. Arch. Allergy Immunol.* (1997) 112:406-411.

54. MATSUBARA S, FUSHIMI K, KAMINURA O *et al.*: **Effect of T-440, a Type IV phosphodiesterase inhibitor on ozone-induced airway hyperresponsiveness and airway epithelial injury in guinea pigs.** *Jpn. J. Pharmacol.* (1996) 71(Suppl. 1):197P.

55. MATSUBARA S, FUSHIMI K, KAMINURA O, KIKKAWA H, IKEZAWA K, NAITO K: **Prevention of ozone-induced airway hyperresponsiveness and epithelial injury by phosphodiesterase inhibitors in guinea pigs.** *Environ. Toxicol. Pharmacol.* (1997) 3:201-209.

56. YAMAGATA S, WADA K, SUGAYA T *et al.*: **Bronchial anti-spasmogenic effects and selectivity of T-440, phosphodiesterase Type 4 inhibitor, in the dog.** *Biol. Pharm. Bull.* (1997) 20(12):1261-1265.

57. MATSUBARA S, KAMINURA O, KIKKAWA H, IKEZAWA K, NAITO K: **Effects of PDE IV inhibitors on antigen-induced bronchoconstriction in rats. Comparision with guinea pigs.** *70th Annual Meeting of the Japanese Pharm. Soc.* (1997):P-550.

58. FUJIMURA M, KITA T, MYOU S, TACHIBANA H, MATSUDA T: **Bronchoprotective effects of KF-19514 and cilostazol in guinea pigs *in vivo*.** *Eur. J. Pharmacol.* (1997) 327:57-63.

59. MANABE H, AKUTA K, SEJIMO H *et al.*: **Anti-inflammatory and bronchodilator properties of KF19514, a phosphodiesterase 4 and 1 inhibitor.** *Eur. J. Pharmacol.* (1997) 332:97-107.

60. MANABE H, AKUTA K, OKAMURA K, OHMORI K: **KF19514, a phosphodiesterase 4 and 1 inhibitor, inhibits PAF-induced lung inflammatory responses by inhaled administration in guinea pigs.** *Int. Arch. Allergy Immunol.* (1997) 114:389-399.

61. HEASLIP RJ, LOMBARDO LJ, GOLANKIEWICZ JM *et al.*: **Phosphodiesterase-IV inhibition, respiratory muscle relaxation and bronchodilation by WAY-PDA-641.** *J. Pharmacol. Exp. Ther.* (1994) 268(2):888-896.

62. HEASLIP RJ, EVANS DY, SICKELS BD: **Pulmonary selectivity of WAY-PDA-641 and reference PDE-IV inhibitors in the dog.** *FASEB J.* (1994) 8:371.

63. HOWELL RE, WOEPPEL SL, HOWELL DE *et al.*: **Pulmonary antiallergic and antiinflammatory effects of a novel, orally-active phosphodiesterase IV inhibitor (WAY-127093B) in guinea pigs and rats.** *Inflamm. Res.* (1995) 44(Suppl. 2):S172-S173.

64. SUZUKI F, KURODA T, KAWAKITA T *et al.*: **New bronchodilators. 3. Imidazo[4,5-c][1,8]naphthyridin-4(5H)-ones.** *J. Med. Chem.* (1992) 35:4866-4874.

65. MATSUURA A, ASHIZAWA N, ASAKURA R *et al.*: **Substituted 1,8-naphthyridin-2(1H)-ones as selective phosphodiesterase IV inhibitors.** *Biol. Pharm. Bull.* (1994) 17(4):498-503.

66. MANABE H, KURODA T, OHNO T *et al.*: **Pharmacological properties of a new bronchodilator, KF17625.** *Jpn. J. Pharmacol.* (1992) 58(Suppl. 1):238P.

67. CAVALLA D, GALE DD, SPINA D *et al.*: **Activity of V11294A, a novel phosphodiesterase 4 (PDE4) inhibitor, in cellular and animal models of asthma.** *Amer. J. Respir. Crit. Care Med.* (1997) 155:A660.

68. TAKAYAMA K, IWATA M, OKAMOTO Y, AOKI M, NIWA A, ISOMURA Y: **Synthetic studies on selective Type IV phosphodiesterase (PDE IV) inhibitors.** *214th ACS Nat. Meeting* (1997) MEDI-245.

69. BELETA BJ: **Characterisation of LAS31025: a new selective PDE IV inhibitor for bronchial asthma.** *Third Int. Conf. on Cyclic Nucleotide Phosphodiesterases: From Genes to Therapies.* Glasgow, UK (1996): Abstract 73.

70. GOZZARD N, HUGHES B, WARRELLOW G, HIGGS G: **Pharmacological characterisation of the neonatally sensitized rabbit model and effects of a novel phosphodiesterase (PDE) 4 inhibitor.** *J. Pharm. Pharmacol.* (1998) 50(Suppl.):69.
- Interesting paper detailing rabbit model.

71. ESCOTT KJ, MCMILLAN SJ, BIRRELL M *et al.*: **Pharmacological profiling of phosphodiesterase 4 (PDE4) inhibitors and analysis of the therapeutic ratio in rats and dogs.** *Br. J. Pharmacol.* (1998) 123(Proc. Suppl.):40P.

72. THURAIRATNAM S, ALDOUS D, BOWER S, ESCOTT KJ, GEIGER LE, IQBAL S: **Biological activity and side effect profile of RPR-132294 and RPR-132703 - novel PDE4 inhibitors.** *XVIth EFMC Int. Symp. Med. Chem.* (1998).

73. MARX D, POPPE H, SZELENYI I: **The *in vivo* activity of AWD 12-281, a potent PDE4 inhibitor for the treatment of allergic asthma.** *J. Allergy Clin. Immunol.* (1999) 103:S127.

74. POPPE H, KUSTERS S, SZELENYI I: **Effect of AWD 12-281, a new selective PDE4-inhibitor, loteprednol and beclomethasone in models of allergic rhinitis and airway inflammation in brown norway-rats.** *Am. J. Respir. Crit. Care Med.* (1999) A95.

75. HOFGEN N, EGERLAND U, POPPE H *et al.*: **Structure based drug design of a new type of selective PDE4 inhibitors.** *XVIth EFMC Int. Symp. Med. Chem.* (1998):Absract P98.

76. HOFGEN N, POLYMEROPOULOS E, EGERLAND U *et al.*: **AWD 12-281, a new PDE4 inhibitor - created by ligand based drug design.** *4th World Congress on Inflammation*, Paris (1999):P-06-5.

77. POPPE H, SZELENYI I: **Effect of the new selective PDE4-inhibitor AWD 12-281 and RPR 73401 on LPS-induced neutrophilia in ferret lung and emetogenic effects in concious ferrets.** *Arch. Pharmacol.* (1999) 359(3):114.

78. BURNOUF C, PASCAL Y, ANDRIANJARA R *et al.*: **Pharmacology of the novel phosphodiesterase Type 4 inhibitor CI-1018.** *215th ACS* Dallas, USA (1998) MEDI 8.

79. PASCAL Y, ANDRIANJARA R, AUCLAIR *et al.*: **Synthesis and structure-activity relationships of 4-oxo-1-phenyl-3,4,6,7-tetrahydro-[1,4]diazepino [6,7,1-hi] indolines: novel PDE4 inhibitors.** *215th ACS* Dallas, USA (1998) MEDI 50.

80. SZELENYI SK, POPPE H, DENT G, MAGNUSSEN H, RABE KF: **Effects of selective PDE4 inhibitor, D-22888, on human airways and eosinophils *in vitro* and late phase allergic pulmonary eosinophilia in guinea pigs.** *Pulmonary Pharmacol. Ther.* (1998) **11**:13-21.

81. MARX D, ROLLING H, SZELENYI I: **Inhibition of allergen induced bronchial hyperactivity (BHR) in BP-2 mice by cyclosporine A, fluticosone and selective PDE4 inhibitors.** *J. Allergy Clin. Immunol.* (1998) **101**:S10.

82. MARX D, EGERLAND U, HARTUNG T, SAUCER A, SZELENYI I, WENDEL A: **D-22888 - a new PDE4 inhibitor for the treatment of allergic rhinitis and other allergic disorders.** *J. Allergy Clin. Immunol.* (1997) **99**:S444.

83. BURNOUF C, PASCAL Y, ANDRIANJARA R, AUCLAIR E, AVENEL N, BERTIN B: **Pharmacology of the novel phosphodiesterase Type 4 inhibitor, CI-1018.** *215th ACS Nat. Meeting* (1998):MEDI 008.

84. PRUNIAUX M-P, ROCHER M-N: **The novel phosphodiesterase inhibitor CI-1018 inhibits antigen-induced lung eosinophilia in sensitised brown-norway rats -comparision with rolipram.** *Inflammation* (1999):S-04-6.

85. STIERNET F, DE LAVELEYE F, DANHAIVE P, PECK MJ, CHATELAIN P, MASSINGHAM R: **UCB29936, a selective phosphodiesterase Type IV inhibitor, suppressed Sephadex-induced bronchial hyperresponsiveness to seratonin in the rat.** *ATS* (1999):B75.

86. MONTANA J, COOPER N, HUNT H *et al.*: **Activity of D4418, a novel phosphodiesterase 4 (PDE4) inhibitor, effects in cellular and animal models of asthma and early clinical studies.** *Am. J. Respir. Crit. Care Med.* (1999):A624.

87. CHENG JB, COOPER K, DUPLANTIER AJ *et al.*: **Synthesis and *in vitro* profile of a novel series of catechol benzimidazoles. The discovery of potent, selective phosphodiesterase Type IV inhibitors with greatly attenuated affinity for the [3H]rolipram binding site.** *Bioorg. Med. Chem. Lett.* (1995) **5**:1969-1972.

88. IWASAKI T, KONDO K, KURODA T *et al.*: **Novel selective PDE IV inhibitors as antiasthmatic agents. Synthesis and biological activities of a series of 1-aryl-2,2-bis(hydroxymethyl)naphthalene lignans.** *J. Med. Chem.* (1996) **39**:2696-2704.

89. DUPLANTIER AJ, BIGGERS MS, CHAMBERS RJ *et al.*: **Biarylcarboxylic acids and -amides: inhibition of phosphodiesterase Type IV *versus* [3H]rolipram binding activity and their relationship to emetic behaviour in the ferret.** *J. Med. Chem.* (1996) **39**:120-125.

90. BACHER E, BOER C, BRAY-FRENCH K *et al.*: **N-Arylrolipram derivatives as potent and selective PDE4 inhibitors.** *Bioorg. Med. Chem. Lett.* (1998) **8**:3229-3234.

91. UKITA T, SUGAHARA M, TERAKAWA Y *et al.*: **Novel, potent, and selective phosphodiesterase-4 inhibitors as antiasthmatic agents: synthesis and biological activities of a series of 1-pyridylnaphthalene derivatives.** *J. Med. Chem.* (1999) **42**:1088-1099.

92. ZELLER E, STIEF HJ, PFLUG B, SASTRE Y, HERNANDEZ M: **Results of a Phase II study of the antidepressant effect of rolipram.** *Pharmacopsychiatry* (1984) **17**:188-190.

93. HOROWSKI R, SASTRE-Y-HERNANDEZ M: **Clinical effects of the neurotropic selective cAMP phosphodiesterase inhibitor rolipram in depressed patients: global evaluation of the preliminary reports.** *Curr. Ther. Res.* (1985) **38**:23-29.

94. HEBENSTREIT GF, FELLERER K, FICHTE K *et al.*: **Rolipram in major depressive disorder: results of a double-blind comparative study with imipramine.** *Pharmacopsychiatry* (1989) **22**:156-160.

95. PUURUNEN J, LUCKE C, SCHWABE U: **Effect of the phosphodiesterase inhibitor 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidone (ZK 62711) on gastric secretion and gastric mucosal cyclic AMP.** *Naunyn-Schmiedeberg's Arch. Pharmacol.* (1978) **304**:69-75.

96. ECKMANN F, FICHTE K, MEYA U, SASTRE-Y-HERNANEZ M: **Rolipram in major depression: results of a double-blind comparative study with amitriptyline.** *Curr. Ther. Res.* (1988) **43**:291-295.

97. WACHTEL H: **Potential antidepressant activity of rolipram and other selective cyclic adenosine 3, 5-monophosphate phosphodiesterase inhibitors.** *Neurpharmacology* (1983) **22**:267-272.

98. BOBON D, BREULET M, GERARD-VANDENHOVE M-A: **Is phosphodiesterase inhibition a new mechanism of antidepressant action?** *Eur. Arch. Psychiatr. Neurol. Sci.* (1988) **238**:2-6.

99. SOUNESS JE, RAEBURN D, KARLSSON J-A: **Phosphodiesterase Type 4 inhibitors as potential anti-asthma agents.** In: *Progress in Basic and Clinical Pharmacology (Anti-asthmatic Drugs)* Pauwels R, Advenier C, O'Bryne P (Eds.) Karger, Basle, Germany. (In Press).

100. NICHOLSON CD: **Cyclic nucleotide phosphodiesterase isoenzymes and asthma - outstanding issues.** *Agents Actions Suppl.* (1993) **43**:3-12.

101. TORPHY TJ, STADEL JM, BURMAN M *et al.*: **Coexpression of human cAMP-specific phosphodiesterase activity and high affinity rolipram binding in yeast.** *J. Biol. Chem.* (1992) **267**:1798-1804.

102. TORPHY TJ, DEWOLF WE, GREEN DW, LIVI GP: **Biochemical characteristics and cellular regulation of phosphodiesterase IV.** *Agents Actions Suppl.* (1993) **43**:51-71.

103. MULLER T, ENGELS P, FOZARD JR: **Subtypes of the Type 4 cAMP phosphodiesterases: structure, regulation and selective inhibition.** *TIPS* (1996) **17**:294-298.

104. JACOBITZ S, MCLAUGHLIN MM, LIVI GP, BURMAN M, TORPHY TJ: **Mapping the functional domains of human recombinant phosphodiesterase 4A: structural requirements for catalytic activity and rolipram binding.** *Mol. Pharmacol.* (1996) **50**:891-899.

105. ROCQUE WJ, HOLMES WD, PATEL IR *et al.*: **Detailed characterisation of a purified Type 4 phosphodiesterase, HSPDE4B2B:differentiation of high- and low-affinity (R)-rolipram binding.** *Protein Express. Purification* (1997) **9**:191-202.

106. SOUNESS JE, RAO S: **Proposal for pharmacologically distinct conformers of PDE4 cyclic AMP phosphodiesterases.** *Cell. Signal.* (1997) **9**(3/4):227-236.
• Interesting paper discussing the high and low affinity binding sites.

107. HUGHES B, OWENS R, PERRY M, WARRELLOW G, ALLEN R: **PDE-4 inhibitors: the use of molecular-cloning in the design and development of novel drugs.** *Drug Discov. Today* (1997) **2**:89-101.
• Good review.

108. BARNETTE MS, CHRISTENSEN SB, UNDERWOOD DC, TORPHY TJ: **Phosphodiesterase 4: biological underpinnings for the design of improved inhibitors.** *Pharmacol. Rev. Commun.* (1997) **8**:65-73.
• Good review.

109. SCHMIECHEN R, SCHNEIDER HH, WACHTEL H: **Close correlation between behavioural response and binding *in vivo* for inhibitors of the rolipram-sensitive phosphodiesterase.** *Psychopharmacology* (1990) **102**:17-20.

110. BARNETTE MS, GROUS M, CIESLINSKI LB, BURMAN M, CHRISTENSEN SB, TORPHY TJ: **Inhibitors of phosphodiesterase IV (PDE IV) increase acid secretion in rabbit isolated gastric glands:correlation between function and interaction with a high affinity rolipram binding site.** *J. Pharmacol. Exp. Ther.* (1995) **273**:1396-1402.

111. BARNETTE MS, MANNING CD, CIESLINSKI M, BURMAN M, CHRISTENSEN SB, TORPHY TJ: **The ability of phosphodiesterase IV inhibitors to suppress superoxide production in guinea-pigs is correlated with inhibition of PDE IV catalytic activity.** *J. Pharmacol. Exp. Ther.* (1995) **273**:674-679.

112. SOUNESS JE, GRIFFIN M, MASLEN C *et al.*: **Evidence that cyclic AMP phosphodiesterase inhibitors suppress TNF-α generation from human monocytes by interacting with a 'low-affinity' phosphodiesterase 4 conformer.** *Br. J. Pharmacol.* (1996) **118**:649-658.

113. BARNETTE MS, O'LEARY BARTUS J, BURMAN M *et al.*: **Association of the anti-inflammatory activity of phosphodiesterase 4 (PDE4) inhibitors with either inhibition of PDE4 catalytic activity or competition for [[3]H]rolipram binding.** *Biochem. Pharmacol.* (1996) **51**:949-956.

114. SOUNESS JE, HOUGHTON C, SARDAR N, WITHNALL MT: **Evidence that cyclic AMP phosphodiesterase inhibitors suppress interleukin-2 release from murine splenocytes by interacting with a 'low-affinity' phosphodiesterase 4 conformer.** *Br. J. Pharmacol.* (1997) **121**:743-750.

115. UNDERWOOD DC, OSBORN RR, NOVAK LB *et al.*: **Inhibition of antigen-induced bronchoconstriction and eosinophil infiltration in the guinea pig by the cyclic AMP-specific phosphodiesterase inhibitor, rolipram.** *J. Pharmacol. Exp. Ther.* (1993) **266**:306-313.

116. DUPLANTIER AJ, TURNER CR: **Novel pharmacological approaches to the treatment of asthma: status and potential of therapeutic classes.** *Drug Discov. Tod.* (1996) **1**(5):199-208.
• Useful review.

117. BRUNNEE T, ENGELSTATTER R, STEINIJANS VW, KUNKEL G : **Bronchodilatory effect of inhaled zardaverine, a phosphodiesterase III and IV inhibitor, in patients with asthma.** *Eur. Respir. J.* (1992) **5**:982-985.

118. NORMAN P: **PDE4 inhibitors 1998.** *Exp. Opin. Ther. Patents* (1998) **8**(7):771-784.
•• Excellent review.

119. BURNOUF C, PRUNIAUX M-P, SZILAGYI CM: **Phosphodiesterase 4 inhibitors.** *Ann. Reports. Med. Chem.* (1998) **33**:91-109.
•• Good review.

120. **LAS-31025.** *Clin. Trials Monitor* (1997) **69**:25-26.

121. NORMAN P: **Arofylline.** *Curr. Opin. Anti-inflamm. Immunomodulatory Invest. Drugs* (1999) **1**:48-50.
•• Very useful review of Almirall's compound.

122. MURDOCH RD, COWLEY H, UPWARD J, WEBBER D, WYLD P: **The safety and tolerability of Ariflo (SB207499), a novel and selective phosphodiesterase 4 inhibitor, in healthy male volunteers.** *Am. J. Respir. Crit. Care Med.* (1998) **157**:A409.

123. NIEMAN RB, FISHER BD, AMIT O, DOCKHORN RJ: **SB207499 (ARIFLO), a second generation, selective oral phosphodiesterase Type 4 (PDE4) inhibitor, attenuates exercise induced bronchoconstriction.** *Am. J. Respir. Crit. Care Med.* (1998) **157**:A413.

124. COMPTON CH, CEDAR E, NIEMAN RB, AMIT O, LANGLEY SJ, SAPENE M: **Ariflo improves lung function in patients with asthma: results of a study in patients taking inhaled corticosteroids.** ALA/ATS (1999).

125. MURDOCH R, COLWEY H, WEBBER D, TAYLOR A: **The addition of SB 207499 (Ariflo), a novel and selective PDE4 inhibitor, to oral prednisolone, is safe and well tolerated.** *Eur. Res. J.* (1998) **12**(Suppl. 28):362s.

126. KELLY J, MURDOCH D, COWLEY H, WEBBER D, TAYLOR AC: **The co-administration of SB 207499 (Ariflo), an orally active, novel and selective PDE4 inhibitor, with oral theophylline does not produce adverse drug interactions in healthy male volunteers.** *Eur. Res. J.* (1998) **12**(Suppl. 28):362s.

127. KELLY J, MURDOCH D, COWLEY H, WEBBER D: **SB 207499 (Ariflo), an orally active, novel second generation PDE4 inhibitor, does not potentiate the cardiovascular effects of nebulised salbutamol when co-administered in healthy male volunteers.** *Eur. Res. J.* (1998) **12**(Suppl. 28):362s.

128. BROWN WM: **SB-207499.** *Curr. Opin. Anti-inflamm. Immunomodulatory Invest. Drugs* (1999) **1**:39-47.
•• Excellent review of SB's compound.

129. HARBINSON PL, MACLEOD D, HAWKSWORTH R *et al.*: **The effect of a novel orally active selective PDE4 isoenzyme inhibitor (CDP840) on allergen-induced responses in asthmatic subjects.** *Eur. Respir. J.* (1997) **10**:1008-1014.
• One of the few papers describing results of a clinical trial of a selective PDE4 inhibitor.

130. HIGGS GA: **The effects of CDP-840 on antigen induced airway responses in pre-clinical and clinical models of asthma.** *212th ACS* (1996):MEDI118.

131. HAWKSWORTH R, HEATH P, LEE TH: **The effects of single doses of CDP840, a potent and selective phosphodiesterase Type IV (PDE IV) inhibitor, on FEV1 in patients with bronchial asthma.** *Am. J. Resp. Crit. Care Med.* (1996) **153**:A347.

132. MACLEOD D, O'TOOLE S, HEATH P, HOLGATE S: **Evaluation of the safety and protection against histamine of a new sekective phosphodiesterase Type IV inhibitor, CDP840, in mild asthma.** *Am. J. Resp. Crit. Care Med.* (1996) **153**:A347.

133. HARBINSON PL, SULLIVAN PJ, WAHID S *et al.*: **Effect of CDP-840, a selective inhibitor of phosphodiesterase Type IV (PDE IV) on the allergen induced asthmatic response.** *Am. J. Resp. Crit. Care Med.* (1996) **153**:A347.

134. **Piclamilast.** *Drugs Future.* (1998) **23**:918-919.
• Useful review of RPR's compound.

135. VACCARO SK, ARGENTI D, SHAH B, GILLEN MS, ROHATAGI S, JENSEN BK: **Effect of food and gender on the pharmacokinetics of RP 73401, a phosphodiesterase IV inhibitor (PDEI).** *J. Clin. Pharmacol.* (1997) **37**:Abstract 28.

136. JONKER GJ, TIJHUIS GJ, DE MONCHY JGR: **RP 73401 (a phosphodiesterase IV inhibitor) single dose does not prevent allergen induced broncho-constriction during the early phase reaction in asthmatics.** *Eur. Respir. J.* (1996) 9(Suppl.):82S.

137. LANDELLS LJ, JENSEN MW, SPINA D *et al.*: **Oral administration of the phosphodiesterase (PDE) 4 inhibitor, V11294A inhibits ex-vivo agonist-induced cell activation.** *Eur. Resp. J.* (1998) **12**(Suppl. 28):362s.

138. GALE DD, LANDELLS D, SPINA AJ *et al.*: **Pharmacodynamic-pharmacokinetic (PD/PK) profile of the phosphodiesterase (PDE0 4 inhibitor, V11294A, in human volunteers.** *ALA/ATS* (1999) B123.

139. Analysts meeting (1998)

140. MACKAY IS: **Classification and differential diagnosis of rhinitis.** *Eur. Respir. Rev.* (1994) **4**:245-247.
• Useful review.

141. NACLERIO RM: **Pathophysiology of perennial allergic rhinitis.** *Allergy* (1997) **52**(Suppl. 36):7-13.
• Useful review.

142. GOMEZ E, CORRADO OJ, BALDWIN DL, SWANTON AR, DAVIES RJ: **Direct *in vivo* evidence for mast cell degranulation during allergen-induced reactions in man.** *J. Allergy Clin. Immunol.* (1986) **78**:673-545.

143. NACLERIO RM, PROUD D, TOGIAS AG *et al.*: **Inflammatory mediators in late antigen-induced rhinitis.** *New Engl. J. Med.* (1985) **313**:65-70.
• Useful review.

144. LIM MC, TAYLOR RM, NACLERIO RM: **The histology of allergic rhinitis and its comparision to cellular changes in nasal lavage.** *Am. J. Respir. Crit. Care Med.* (1995) **151**:136-144.

145. MOHAMMED S, YOPUNG A: **Clinical aspects and treatment of chronic obstructive pulmonary disease.** *Curr. Opin. Anti-inflamm. Immunomodulatory Invest. Drugs* (1999) **1**:21-28.
•• Very useful review.

146. PRIDE NB, BURROWS B: **Development of impaired lung function: natural history and risk factors.** *Chronic Obstructive Pulmonary Disease* Calverly P, Pride N (Eds) Chapman & Hall Medical, London (1995):47-67.

147. SANDFORD AJ, WEIR TD, PARE PD: **Genetic risk factors for chronic obstructive pulmonary disease.** *Eur. Respir. J.* (1997) **10**:1380-1391.

148. CELLI BR, NIDER GL, HEFFNER J *et al.*: **Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease.** *Am. J. Repir. Crit. Care Med.* (1995) **152**:S77-S120.

149. MURRAY CJL, LOPEZ A: **Evidence-based health policy-lessons from the global burden of disease study.** *Science* (1996) **274**:740-743.

150. ANTHONISEN NR, CONNETT JE, KILEY JP *et al.*: **Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV1. The Lung Health Study.** *J. Am. Med. Assoc.* (1994) **272**:1497-1505.

151. BARNES PJ: **Chronic obstructive pulmonary disease: new opportuniries for drug development.** *TiPS* (1998) **19**:415-423.
•• An informative review about existing and new treatments.

152. CULPITT S, MAZIAK W, LOUKIDIS S, KELLER A, BARNES PJ: **Effect of theophylline on induced sputum inflammatory indices in COPD patients.** *Am. J. Respir. Crit. Care Med.* (1998) **157**:A797.

153. WRIGHT CD, KUIPERS PJ, KOBYLARZ SINGER D, DEVALL LJ, KLINEFUS BA, WEISHAAR RE: **Differential inhibition of human neutrophil functions: role of cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase.** *Biochem. Pharmacol.* (1990) **40**:699-707.

154. SCHUDT C, WINDER S, FORDERKUNZ S, HATZELMANN A, ULLRICH V: **Influence of selective phosphodiesterase inhibitors on human neutrophil functions and levels of cAMP and Cai.** *Naunyn Schmiedebergs Arcb. Pharmacol.* (1991) **344**:682-690.

155. TENOR H, HATZELMANN A, KUPFERCSHMIDT R *et al.*: **Cyclic nucleotide phosphodiesterase isoenzyme activities in human alveolar macrophages.** *Clin. Exp. Allergy* (1995) **25**:625-633.

156. NIELSON CP, VESTAL RE, STURM RJ, HEASLIP R: **Effect of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte respiratory burst.** *J. Allergy Clin. Immunol.* (1990) **86**:801-808.

157. BLOEMAN PGM, VAN DEN TWEEL MC, HENRICKS PAJ *et al.*: **Increased cAMP levels in stimulated neutrophils inhibit their adhesion to human broncial epithelial cells.** *Am. J. Pystol.* (1997) **272**:L580-587.

158. AL ESSA L, NIWA M, KOHNO K, NOZAKI M, TSURUMI K: **Heterogeneity of circulating and exudated polymorphonuclear leukocytes in superoxide-generating response to cyclic AMP and cyclic AMP-elevating agents: investigation of the underlying mechanism.** *Biochem. Pharmacol.* (1995) **49**:315-322.

159. OTTONELLO L, MORONE MP, DAPINO P, DALLEGRI F: **Cyclic AMP-elevating agents down-regulate the oxidative burst induced by granulocyte-macrophage colony-stimulating (GM-CSF) in adherent neutrophils.** *Clin. Exp. Immunol.* (1995) **101**:502-506.

160. OTTONELLO L, MORONE MP, DAPINO P, DALLEGRI F: **Tumour necrosis factor alpha-induced oxidative burst in neutrophils adherent to fibronectin: effects of cyclic AMP-elevating agents.** *Br. J. Haematol.* (1995) **91**:566-570.

161. BERENDS C, DIJKHUIZEN B, DEMONCHY JGR, DUBOIS AEJ, GERRITSEN J, KAUFFMAN HF: **Inhibition of PAF-induced expression of CD11b and shedding of L-selectin on human neutrophils and eosinophils by the type-IV selective PDE inhibitor, rolipram.** *Eur. Respir. J.* (1997) **10**:1000-1007.

162. DERIAN CK, SANTULLI RJ, RAO PE, SOLOMAN HF, BARRETT JA: **Inhibition of chemotactic peptide-induced neutrophil adhesion to vascular endothelium by cAMP modulators.** *J. Immunol.* (1995) **154**:308-317.

163. SCHUDT C, TENOR H, HATZELMANN A: **PDE isoenzymes as targets for anti-asthma drugs.** *Eur. Respir. J.* (1995) **8**:1179-1183.

164. KAMBAYASHI T, JACOB CO, ZHOU D, MAZUREK N, FONG M, STRASSMANN G: **Cyclic nucleotide phosphodiesterase Type IV participates in the regulation of IL-10 and the subsequent inhibition of TNF-alpha and IL-6 release by endotoxin-stimulated macrophages.** *J. Immunol.* (1995) **155**:4909-4916.

165. KAMINUMA O, MURAKAMI S, SUKO M *et al.*: **Late airway obstruction and neutrophil infiltration in sensitised mice after antigen provocation were suppressed by selective and non-selective phosphodiesterase inhibitors.** *All. Int.* (1997) **46**:101-108.

166. UNDERWOOD DC, OSBORN RR, BOCHNOWICZ S *et al.*: **The second generation phosphodiesterase (PDE) 4 inhibitor, SB 207499, inhibits antigen-induced bronchoconstriction and eosinophilia and LPS-induced airway neutrophilia and edema in the guinea-pig.** *Eur. Respir. J.* (1998) **12**:86s.

167. COMPTON CH, GUBB J, CEDAR E *et al.*: **The efficacy of Ariflo (SB 207499), a second-generation, oral PDE4 inhibitor in patients with COPD.** *ALA/ATS* (1999).

168. BADGER AM, LEE JC: **Advances in antiarthritic therapeutics.** *Drug Discov. Today* (1997) **2**:427-435.
•• An informative review about existing and new treatments.

169. HARRIS ED, JR. : **Rheumatoid arthritis. Pathophysiology and implications for therapy.** *New Engl. J. Med.* (1990) **322**:1277-1289.

170. GAY S, GAY RE, KOOPMAN WJ: **Molecular and cellular mechanisms of joint destruction in rheumatoid arthritis: two cellular mechanisms explain joint destruction.** *Ann. Rheum. Dis.* (1993) **52**:S39-S47.

171. BROOKES PM: **Clinical management of rheumatoid arthritis.** *Lancet* (1993) **341**:286-290.

172. CONAGHAN PG, BROOKS P: **Disease modifying anti-rheumatic drugs.** *Curr. Opin. Rheumatol.* (1996) **8**:176-182.

173. PINCUS T : **The paradox of effective therapies, but poor long term outcomes in rheumatoid arthritis.** *Arth. Rheum.* (1992) **21**:2-15.

174. MILLER LADNER U : **Molecular and cellular interactions in the rheumatoid synovium.** *Curr. Opin. Rheumatol.* (1996) **8**:210-220.

175. FIRESTEIN GS, ZVAIFLER WJ : **How important are T-cells in chronic rheumatoid synovitis?** *Arth. Rheum.* (1990) **33**:768-773.

176. MAINI RN, ELLIOTT MJ, BRENNAN M, FELDMAN M: **Beneficial effects of tumour necrosis factor-alpha (TNF-α) blockade in rheumatoid arthritis (RA).** *Clin. Exp. Immunol.* (1995) **101**:207-212.
• An interesting paper on the biological approach to TNF blockade.

177. PALFREYMAN MN, SOUNESS JE: **Phosphodiesterase Type IV inhibitors.** *Prog. Med. Chem.* (1996) **33**:1-52.

178. COHAN VL, SHOWELL HJ, FISHER DA *et al.*: ***In vitro* pharmacology of the novel phosphodiesterase Type IV inhibitor, CP80-633.** *J. Pharm. Exp. Ther.* (1996) **278**:1356-1361.

179. BARNETTE MS, CHRISTENSEN SB, ESSAYAN DM *et al.*: **SB207499 (Ariflo), a potent and selective second generation phosphodiesterase 4 inhibitor: *in vitro* antiinflammatory actions.** *J. Pharm. Exp. Ther.* (1998) **284**:420-426.

180. KAMBAYASHI T, JACOB W, ZHON D, MAZUREK N, FONG STRASSMAN G: **Cyclic nucleotide phosphodiesterase Type 4 participates in the regulation of IL10 and the subsequent inhibition of TNF-α and IL6 release by endotoxin stimulated macrophages.** *J. Immunol.* (1995) **155**: 4909-4916.

181. LAPADULA G, IANNOS F, DELL'ACCIO F, COVELLI M, PIPITONE V: **Interleukin 10 in rheumatoid arthritis.** *Clin. Exp. Rheumatol.* (1995) **13**:629-632.

182. CHENG JB, WATSON JW, PAZOLES CJ *et al.*: **The phosphodiesterase Type 4 (PDE4) inhibitor CP-80,633 elevates cyclic AMP levels and decreases TNFα production in mice: effect of adrenalectomy.** *J. Pharm. Exp. Ther.* (1997) **280**:621-626.

183. SINGH HN, BLANCUZZI V, GREENWOOD S, SKILES JW, O'BYRNE EM: **Synovial fluid levels of tumour necrosis factor a in the inflamed rat knee: Modulation by dexamethasone and inhibitors of matrix metalloproteinases and phosphodiesterases.** *Inflamm. Res.* (1997) **46** (Suppl. 2):S153-S154.

184. SEKUT L, YARNALL D, STIMPSON SA *et al.*: **Antiinflammatory activity of phosphodiesterase (PDE) IV inhibitors in acute and chronic models of inflammation.** *Clin. Exp. Immunol.* (1995) **100(1)**:126-132.

185. NYMAN U, MUSSENER A, LARSSON E, LORENTZEN J, KLARESKOG L: **Amelioration of collagen II induced arthritis in rats by the Type IV phosphodiesterase inhibitor rolipram.** *Clin. Exp. Immunol.* (1997) **108**:415-419.

186. ROSS S, WILLIAMS RO, MASON LJ *et al.*: **Amelioration of established murine collagen-induced arthritis following treatment with rolipram.** *Br. J. Rheumatol.* (1996) **35**:Abstract Suppl. 1.

187. SOUNESS JE, FOSTER M: **Potential of phosphodiesterase Type IV inhibitors in the treatment of rheumatoid arthritis.** *Drugs* (1998) **1**(5):541-553.

188. HULME C, METHEW R, MORIARTY K *et al.*: **Orally active indole-N-oxide PDE4 inhibitors.** *Bioorg. Med. Chem. Letts.* (1998) **8**(21):3053-3058.

189. REGAN J, BRUNO J, McGARRY D *et al.*: **2-Substituted-4-methoxy benzimidazole based PDE4 inhibitors.** *Bioorg. Med. Chem. Letts.* (1998) **8**(19):2737-2742.

190. CHIKANZA IC, JAWED S, PERROT S *et al.*: **The clinical effects of RP73401 phosphodiesterase Type 4 inhibitor in patients with rheumatoid arthritis.** *Br. J. Rheumatol.* (1997) **36**:Abstr. Suppl. I, 186.

191. CHIKANZA IC, JAWED SJ, BLAKE DR *et al.*: **Treatment of patients with rheumatoid arthritis with RP73401 phosphodiesterase Type IV inhibitor.** *Arth. Rheum.* (1996) **39**(9):S282.

192. MONTANA J, BIRD J: **New therapeutic approaches to atopic dermatitis.** *Exp. Opin. Invest. Drugs.* (1996) 5(9):1173-1180.
- Provides a good overview of existing and potential therapies.

193. HANIFIN JM, RAJKA G : **Diagnostic features of atopic dermatitis.** *Acta Derm. Venerol.* (1980) **92**:44-47.

194. LEUNG DYM: *Atopic Dermatitis: From Pathogenesis To Treatment.* RG Landes Co. Austin, Texas, USA (1996):1-226.
•• An informative review.

195. BOGUNIEWICZ M: **Advances in the understanding of atopic dermatitis.** *Curr. Opin. Pediatrics.* (1997) 9(6):577-581.

196. CROCKER IC, TOWNLEY RG, KHAN MM: **Phosphodiesterase inhibitors suppress proliferation of peripheral blood mononuclear cells, and IL4 and IL5 secretion by human T-helper Type 2 cells.** *Immunopharmacology* (1996) **31**(2-3):223-235.

197. LEUNG DYM, DIAZ LA, DeLEO V, SOTER NA: **Allergic and immunological skin disorders.** *JAMA* (1997) **278**(22):1914-1923.

198. BOURNE HR, LICHTENSTEIN LM, HENNEY CS: **Effects of cholera toxin on *in vitro* models of immediate and delayed type hypersensitivity: further evidence for the role of cAMP.** *J. Clin. Invest.* (1973) **52**:691-697.

199. GANTNER F, GOTZ C, GEKENELER V, SCHUDT C, WENDEL A, HATZELMANN : **Phosphodiesterase profile of human B lymphocytes from normal and atopic donors and the effects of PDE inhibitors on B-cell proliferation.** *Br. J. Pharmacol.* (1998) **123**(6):1031-1038.

200. BANNER KH, ROBERTS NM, PAGE CP: **Differential effect of phosphodiesterase 4 inhibitors on the proliferation of human peripheral blood mononuclear cells from normals and subjects with atopic dermatitis.** *B. J. Pharmacol.* (1995) **116**(8):3169-3174.

201. CHAN SC, HANIFIN JM: **Differential inhibitory effects of cAMP phosphodiesterase isoforms in atopic and normal leukocytes.** *J. Lab. Clin. Med.* (1993) **121**(1): 44-51.
- Provides insight into the potential differences between atopic and normal PDE4.

202. MONTANA JG, COOPER N, DYKE HJ *et al.*: **Phosphodiesterase 4 inhibitors: new xanthine analogues.** *Bioorg. Med. Chem. Letts.* (1998) **8**:2925-2930.

203. MONTANA JG, BUCKLEY GM, COOPER N *et al.*: **Aryl sulphonamides as selective phosphodiesterase 4 inhibitors.** *Bioor. Med. Chem. Letts.* (1998) **8**:2635-2640.

204. BEASLEY SC, COOPER N, GOWERS L *et al.*: **Synthesis and evaluation of a novel series of phosphodiesterase 4 inhibitors. A potential treatment for asthma.** *Bioorg. Med. Chem. Letts.* (1998) **8**:2629-2634.

205. HANIFIN JM, CHAN SC, CHENG JB, HENDERSON Jr WR: **Type 4 phosphodiesterase inhibitors have clinical and *in vitro* anti-inflammatory effects in atopic dermatitis.** *J. Invest. Dermatol.* (1996) **107**:51-56.

206. NAGPAL S, CHANDRARATNA RAS: **New dermatological agents for the treatment of psoriasis.** *Ann. Rep. Med. Chem.* (1997) **32**:201-210.
- An informative review about existing and new treatments.

207. BAADSGAARD O: **The role of the immune system in the pathogenesis of psoriasis.** *J. Invest. Dermatol.* (1990) **95**:32S-34S.

208. ANDERSON TF: **Psoriasis.** *Med. Clin. North Am.* (1982) **66**(4):769-794.

209. DUELL EA: **Adenosine induced alterations in the adenosine 3'5'-monophosphate levels in mammalian epidermis.** *Mol. Pharmacol.* (1980) **18**(1):49-52.

210. RUSTIN LJ, DUELL EA, VOORHEES JJ: **Papaverine and Ro20-1724 inhibit cyclic nucleotide phosphodiesterase activity and increase cAMP levels in psoriatic epidermis *in vitro*.** *J. Invest. Dermatol.* (1978) **71**(2):154-156.

211. KRAUSE W, KUHNE G: **Anti-inflammatory activity of rolipram in a rat ear edema model.** *Arzneim-Forsch Drug Res.* (1994) **44**(1):163-165.

212. STAWISKI MA, RUSTIN LJ, BURNS TL: **Ro20-1724 An agent that significantly improves psoriatic lesions in double blind clinical trials.** *J. Invest. Dermatol.* (1979) **73**(4):261-263.

213. RAINE CS: **The Dale E. McFarlin Memorial Lecture: the immunology of the multiple sclerosis lesion.** *Ann. Neurol.* (1994) **36**:S61-72.

214. LUCCHINETTI CF, BRUCK W, RODRIGUEZ M *et al.*: **Distinct patterns of multiple sclerosis pathology indicates heterogeneity on pathogenesis.** *Brain Pathol.* (1996) **6**:259-274.

215. BRUCK W, BITSCH A, KOLENDA H *et al.*: **Inflammatory central nervous system demylination; correlation of magnetic resonance imaging findings with lesion pathology.** *Ann. Neurol.* (1997) **42**:783-93.

216. STORCH M, LASSERMAN H: **Pathology and pathogenesis of demylinating disease.** *Curr. Opin. Neurol.* (1997) **10**:186-192.

217. MILLER DH, GROSSMAN RI, REINGOLD SC *et al.*: **The role of magnetic resonance techniques in understanding and managing multiple sclerosis.** *Brain* (1998) **121**:3-24.

218. WEILBACH FX, GOLD R: **Disease modifying treatments for multiple sclerosis. What is on the horizon?** *CNS Drugs* (1999) **11**:133-157.
• A useful review of new treatments.

219. OZAWA K, SUCHANEK G, BREITSCHOPF H *et al.*: **Patterns of oligodendroglia pathology in multiple sclerosis.** *Brain* (1994) **117**:1311-1322.

220. TRAUGOTT U, RENHERZ EL, RAINE CS: **Multiple sclerosis: distribution of T-cell subsets within active chronic lesions.** *Science* (1984) **219**:308-310.

221. CALDER V, OWEN S, WATSON C, FELDMAN M, DAVIDSON A: **MS: a localised immune disease of the central nervous system.** *Immunol. Today* (1989) **10**:99-103.

222. FUJINAMI RS, OLDSTONE MB: **Amino acid homology between the encephalitogenic site of mylin basic protein and virus: mechanism for autoimmunity.** *Science* (1985) **230**:1043-1045.

223. SELMAJ K, RAINE CS, CANELLA B, BROSNAN CF: **Identification of lymphotoxin and tumour necrosis factor in multiple sclerosis lesions.** *J. Clin. Invest.* (1991) **87**:949-954.

224. WOODROOFE MN, CUZNER ML: **Cytokine mRNA expression in inflammatory multiple sclerosis lesions: detection by non-radioactive *in situ* hybridisation.** *Cytokine* (1993) **5**:583-588.

225. SELMAJ KW, RAINE CS: **Tumor necrosis factor mediates mylin and oligodendrocyte damage *in vitro*.** *Ann. Neurol.* (1988) **23**:339-346.

226. HURWITZ AA, LYMAN WD, GUIDA MP, CALDERON TM, BERMAN JW: **Tumor necrosis factor α induces adhesion molecule expression on human fetal astrocytes.** *J. Exp. Med.* (1992) **176**:1631-1636.

227. SHARIEF MK, THOMPSON EJ: ***In vivo* relationship of tumor necrosis factor-a to blood-brain barrier damage in patients with active multiple sclerosis.** *J. Neuroimmunol.* (1992) **38**:27-34.

228. RUDDLE NH, BERGMAN CM, MCGRATH KM *et al.*: **An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis.** *J. Exp. Med.* (1990) **172**:1193-1200.

229. SELMAJ K, RAINE CS, CROSS AH: **Anti-tumor necrosis factor therapy abrogates autoimmune demyelination.** *Ann. Neurol.* (1991) **30**:694-700.

230. SELMAJ K, PAPIERZ W, GLABINSKI A, KOHNO T: **Prevention of chronic relapsing experimental autoimmune encephalomyelitis by soluble tumor necrosis Factor I.** *J. Neuroimmunol.* (1995) **56**:135-141.

231. REENO T, KRAKOWSKI M, PICCIRILLO C *et al.*: **TNF-alpha expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis: regulation by Th1 cytokines.** *J. Immunol.* (1995) **154**:944-953.

232. PROBERT L, AKASSOGLOU K, PASPARAKIS M *et al.*: **Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-spewcific expression of tumor necrosis factor alpha.** *Proc. Natl. Acad. Sci. USA* (1995) **92**:11294-11298.

233. LIU J, MARINO MW, WONG G *et al.*: **TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination.** *Nature Med.* (1998) **4**:78-83.

234. DINTER H, ONUFFER J, FAULDS D, PEREZ HD: **Phosphodiesterase Type IV inhibitors in the treatment of multiple sclerosis.** *J. Mol. Med.* (1997) **75**:95-102.
• A short review on the potential of PDE4 inhibitors in MS.

235. SOMMER N, LOSCHMANN P-A, NORTHOFF GH *et al.*: **The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis.** *Nature Med.* (1995) **1**:244-248.

236. JUNG S, ZIELASEK J, KOLLNER G *et al.* **Preventive but not therapeutic application of rolipram ameliorates experimental autoimmune encephalomyelitis in Lewis rats.** *J. Neuroimmunol.* (1996) **68**:1-11..

237. GENAIN CP, ROBERTS T, DAVIS RL *et al.*: **Prevention of autoimmune demyelination in non-human primates by a cAMP-specific phosphodiesterase inhibitor.** *Proc. Natl. Acad. Sci. USA* (1995) **92**:3601-3605.

238. SOMMER N, MARTIN R, MCFARLAND HF *et al.*: **Therapeutic potential of phosphodiesterase Type 4 inhibition in chronic autoimmune demyelinating disease.** *J. Neuroimmunol.* (1997) **79**:54-61.
• A short review on the potential of PDE4 inhibitors in MS.

239. *Scrip.* (1997) 2228:10.

240. COMPANY COMMUNICATION (1999).

241. BICKSTON SJ, CAMINELLI F: **Recent developments in the medical therapy of IBD.** *Curr. Opin. Gastroenterol.* (1998) **14**:6-10.
•• Provides a good overview of this therapeutic area.

242. MURTHY S, FLANIGAN A, OSBOURNE BJ, MURTHY NS: **Inflammatory bowel disease: A new wave of therapy.** *Exp. Opin. Ther. Patents.* (1998) **8**(7):785-818.
•• Provides a good overview of this therapeutic area.

243. SHIVANANDA S, LENNARD-JONES J, LOGAN RFA: **Incidence of inflammatory bowel disease across**

Europe: Is there a difference between north and south? *Gut* (1996) 39:690-697.

244. HANAVER SB: **Aminosalicylate therapy for IBD.** In: *Inflammatory Bowel Diseases* Rachmilewitz D (Ed), Kulwer Academic Press (1994):192-197.

245. LOFBERG R: **New steroids for inflammatory bowel disease.** *Inflamm. Bowel Dis.* (1995) 1:135-141.

246. ROSENBERG JL, LEVIN B, WALL AJ: **A controlled trial of azathioprine in Crohns disease.** *Am. J. Dig. Dis.* (1975) 20:721-726.

247. PRESENT DH, MELTZER SJ, KRUMHOLTZ MP: **6-Mercaptopurine in the management of inflammatory bowel disease: short and long term toxicology.** *Ann. Intern. Med.* (1989) 111:641-649.

248. FEAGAN BG, ROCHON J, FEDORAK RN: **Methotrexate for the treatment of Crohns disease.** *New Engl. J. Med.* (1995) 332:292-297.

249. RADFORD-SMITH G, JEWELL DP: **Cytokines and inflammatory bowel disease.** *Baillieres Clin. Gasteroenterol.* (1996) 10:151-164.

250. BRAEGGER CP, NICHOLLS S, MURCH SH: **Tumour necrosis factor in the stool as a marker of intestinal inflammation.** *Lancet* (1992) 339:89-91.

251. PUIG J, SALCEDO C, SALAS A, FERNANDEZ AG, PALACICOS JM: **Curative effects of phoshodiesterase 4 inhibitors in dextran sulphate. Sodium induced colitis in the rat.** *Gastroententology* (1998) 114(4): A1064.

252. CARDELUS I, GRAS J, JAUREGUI J, LLENAS J, PALACIOS JM: **Inhibiting LPS induced bowel erythrocyte extravasation in rats, and of mesenteric hypoperfusion in dogs, by phosphodiesterase inhibitors.** *Eur. J. Pharmacol.* (1996) 299:153-159.

253. CARDELUS I, GRAS J, PUIG J, LLENAS J, PALACIOS J M: **Protective effects of denbufylline against endotoxin induced bowel hyperplasia.** *Met. Find. Exp. Clin. Pharamcol.* (1995) 17(Suppl. A):142

254. DICKIE BGM, GREENFIELF SA: **Chronic exposure to Ro20-1724 protects dopaminergic neurons *in vitro* against the neurotoxic action of N-methyl-D-aspartate and 1-methyl-4-phenylpyridinium.** *Brain Res.* (1997) 753:335-339.

255. HULLEY P, HARTIKKA J, ABDEL'AL S *et al.*: **Inhibitors of Type IV phosphodiesterases reduce the toxicity of MPTP in substantia nigra neurons *in vivo*.** *Eur. J. Neurosci.* (1995) 7:2431-2440.

256. EGAWA T, MISHIMA K, MATSUMOTO Y, IWASAKI K, IWASAKI K, FUJIWARA M: **Rolipram and its optical isomers, phosphodiesterase 4 inhibitors, attenuated the scopolamine-induced impairments of learning and memory in rats.** *Jpn. J. Pharmacol.* (1997) 75:275-281.

257. IMANISHI T, SAWA A, ICHIMARU Y *et al.*: **Ameliorating effects of rolipram on experimentally induced impairments of learning and memory in rodents.** *Eur. J. Pharmacol.* (1997) 321:273-278.

258. BARAD M, BOURTCHOULADZE R, WINDER DG, GOLAN H, KANDEL E: **Rolipram, a Type IV-specific phosphodiesterase inhibitor, facilitates the establishment of long-lasting long-term potentiation and improves memory.** *Proc. Natl. Acad. Sci. USA* (1998) 95:15020-15025.

259. YAMASHITA N, MIYASHIRO M, BABA J, SAWA A *et al.*: **Rolipram, a selective inhibitor of phosphodiesterase Type 4, pronouncedly enhanced the forskolin-induced promotion of dopamine biosynthesis in primary cultured rat mesencephalic neurons.** *Jpn. J. Pharmacol.* (1997) 75:91-95.

260. BARNARD JW, SEIBERT AF, PRASAD VR *et al.*: **Reversal of pulmonary capillary ischemia-reperfusion injury by rolipram, a cAMP phosphodiesterase inhibitor.** *J. Appl. Physiol.* (1994) 77:774-781.

261. KATO H, ARAKI T, ITOYAMA Y, KOGURE K: **Rolipram, a cyclic AMP-selective phosphodiesterase inhibitor, reduces neuronal damage following cerebral ischemia in the gerbil.** *Eur. J. Pharmacol.* (1995) 272:107-110.

262. BLOCK F, TONDAR A, SCHMIDT W, SCHWARZ M: **Delayed treatment with rolipram protects against neuronal damage following global ischemia in rats.** *NeuroReport* (1997) 8:3829-3832.

263. BELAYEV L, BUSTO R, IKEDA M *et al.*: **Protection against blood-brain barrier disruption in focal cerebral ischemia by the Type IV phosphodiesterase inhibitor BBB022: a quantitative study.** *Brain Res.* (1998) 787:277-285.

264. BEGANY DP, CARCILLO JA, HERZER WA, MI Z, JACKSON EK: **Inhibition of Type IV phosphodiesterase by Ro-20-1724 attenuates endotoxin-induced acute renal failure.** *J. Pharmacol. Exp. Ther.* (1996) 278:37-41.

265. MIYAMOTO K, WAKI Y, HORITA T, KASUGAI S, OHYA K: **Reduction of bone loss by denbufylline, an inhibitor of phosphodiesterase 4.** *Biochem. Pharmacol.* (1997) 54:613-617.

266. WAKI Y, HORITA T, MIYAMOTO K, OHYA K, KASUGAI S: **Effects of XT-44, a phosphodiesterase 4 inhibitor, in osteoblastgenesis and osteoclastgenesis in culture and its therapautic effects in rat osteopenia models.** *Jpn. J. Pharmacol.* (1999) 79:477-483.

267. CARDELUS I, GRAS J, JAUREGI J, LLENAS J, PALACIOS JM: **Inhibition of lipopolysaccharide-induced bowel erythrocyte extravasation in rats, and of mesenteric hypoperfusion in dogs, by phosphodiesterase inhibitors.** *Eur. J. Pharmacol.* (1996) 299:153-159.

268. TEIXEIRA MM, GRISTWOOD RW, COOPER N: **Phosphodiesterase (PDE)4 inhibitors: anti-inflammatory drugs of the future?** *TiPS* (1997) 18:164-171.

269. DOUSA MK, MOORE SB, PLOEGER NA, DEGOEY SR, DOUSA TP: **Antagonists of cyclic nucleotide phosphodiesterase (PDE) isozymes PDE3 and PDE4 suppress lymphoblastic response to HLA class II alloantigens: a potential novel approach to preventing allograft rejection?** *Clin. Neph.* (1997) 47:187-189.

270. LIANG L, BESHAY E, PRUD'HOMME GJ: **The phosphodiesterase inhibitors pentoxifylline and rolipram prevent diabetes in NOD mice.** *Diabetes* (1998) 47:570-575.

271. XIANG M, ZACCONE P, DI MARCO R *et al.*: **Prevention by rolipram of concanavalin A-induced T-cell-dependent hepatitis in mice.** *Eur. J. Pharmacol.* (1999) 367:399-404.

272. XU H, STRASSMAN G, CHAN CC *et al.*: **Protective effect of the Type IV phosphodiesterase inhibitor rolipram in EAU: protection is independent of the IL-10-inducing activity.** *Invest. Ophthalmol. Visual Sci.* (1999) 40:942-950.

273. KIM DH, LERNER A: **Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia.** *Blood* (1998) 92:2484-2494.

274. ANGEL JB, SAGET BM, WALSH SP *et al.*: **Rolipram, a specific Type IV phosphodiesterase inhibitor, is a potent inhibitor of HIV-1 replication.** *AIDS* (1995) 9:1137-1144.

275. NAVARRO J, PUNZON C, JIMENEZ JL *et al.*: **Inhibition of phosphodiesterase Type IV suppresses human immunodeficiency virus Type 1 replication and cytokine production in primary T cells: involvement of NF-kappaB and NFAT.** *J. Virol.* (1998) 72:4712-4720

Patents

301. MEIJI SEIKA KAISHA LTD.: WO9528177 (1995).

302. MEIJI SEIKA KAISHA LTD.: JP9221423 (1997).

303. UNIV. OF PITTSBURGH: WO9800135 (1998).

304. MIYAMOYO: JP9169665 (1997).

305. FUJISAWA PHARM. CO. LTD.: JP10067682 (1998)

306. SCHERING AG: DE4230755 (1994).

307. SCHERING AG: WO9406423 (1994).

308. PORSSMANN W-G: WO9902161 (1999).

309. STIEF CG: WO9902161 (1999).

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Suppression of TNF-α Expression, Inhibition of Th1 Activity, and Amelioration of Collagen-Induced Arthritis by Rolipram[1]

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Rolipram is a type IV phosphodiesterase inhibitor that suppresses inflammation and TNF-α production. As anti-TNF-α therapy is effective in rheumatoid arthritis, we investigated the effect of rolipram on collagen-induced arthritis (CIA), a murine model of rheumatoid arthritis. Rolipram was administered after the onset of clinical arthritis at doses of 0.5, 3, 5, or 10 mg/kg twice daily, with a dose-dependent therapeutic effect on clinical severity and joint erosion. Immunohistochemical analysis of joints of rolipram-treated mice revealed 67% reduction in TNF-α-expressing cells compared with control arthritic mice. In vitro studies using bone marrow-derived macrophages confirmed that rolipram directly suppressed TNF-α and IL-12 production following stimulation with IFN-γ and LPS. The effect of rolipram on T cell activity was studied by measuring Th1/Th2 cytokine production by collagen-stimulated draining lymph node cells from arthritic mice treated in vivo with rolipram. Rolipram reduced IFN-γ production and increased IL-10, indicating that rolipram down-regulated the ongoing Th1 response to type II collagen. Finally, the effect on CIA of combination therapy was studied using rolipram plus either anti-TNF-α or anti-CD4 mAbs. Rolipram plus anti-TNF-α was not therapeutically additive, whereas rolipram plus anti-CD4 mAb was clearly additive. This result indicates that the therapeutic effects of rolipram overlap with TNF-α blockade, but are complementary to anti-CD4 treatment. It is therefore proposed that a major mechanism of action of rolipram in CIA is suppression of TNF-α activity. These findings suggest that type IV phosphodiesterase inhibitors may be effective in pathologic conditions, such as RA, with overexpression of TNF-α. *The Journal of Immunology,* **1997, 159: 6253–6259.**

Rheumatoid arthritis (RA)[4] is a debilitating disease of unknown aetiology involving chronic inflammation in the synovial membranes of the joint, accompanied by erosion of cartilage and bone. A number of previous reports documented the abundant presence of TNF-α- and IL-1-producing cells in the joints of RA patients, thereby providing evidence for the involvement of these proinflammatory cytokines in the pathogenesis of the disease (1, 2). In additional studies, it was demonstrated that neutralization of TNF-α in RA synovial cell cultures led to decreased production of other proinflammatory cytokines, including IL-1 and granulocyte-macrophage CSF, indicating that TNF-α plays an important role in promoting inflammation in the joint (3–6). Further evidence supporting this concept came from a number of studies in collagen-induced arthritis (CIA), an animal model of RA, in which administration of TNF-α to mice during the induction phase of the disease accelerated the onset of arthritis and increased its severity (7–9), whereas TNF-α-blockade at this time resulted in arthritis of reduced severity (10, 11). We were subsequently able to demonstrate that anti-TNF-α mAb treatment reduced clinical scores, paw swelling, and joint damage, even when administered after the onset of clinical arthritis (10). It was subsequently shown in clinical trials that treatment with a chimeric anti-TNF-α mAb (cA2) had a clear ameliorative effect on human RA, as judged by highly significant reductions in clinical and laboratory-based indices of disease severity (12). These findings confirm two important points: first, that anti-TNF-α treatment is highly effective in human RA, and second, that the CIA model can be reliably used for predicting the likely effects of different TNF-α-targeted therapeutic strategies.

A potential disadvantage of using chimeric or humanized mAbs (containing murine epitopes) is that repeated treatment may evoke a neutralizing Ab response, which in the course of time may render the treatment less effective (13). For this reason, attention has now turned to the possible use of synthetic, small molecular weight compounds capable of inhibiting the production of TNF-α. For example, rolipram, a type IV phosphodiesterase (PDE IV) inhibitor (14–18), has recently been shown to down-regulate the production of TNF-α by lymphocytes and macrophages (19, 20). Inhibition of PDE IV results in increased levels of intracellular cAMP, an important second messenger thought to be involved in regulating the production not only of TNF-α (21) but also of reactive oxygen species (22, 23), nitric oxide (24), and lipid mediators of inflammation. In addition, raised levels of cAMP are associated with reduced cellular proliferation, motility, chemotaxis, and migration to sites of inflammation (25).

The therapeutic potential of PDE IV inhibitors in autoimmune conditions has recently been highlighted by studies in experimental allergic encephalomyelitis, a model of multiple sclerosis, in which significant reductions in the severity of disease were reported following treatment with rolipram (26, 27). The aim of the present study is to investigate the effect of rolipram in established CIA, as a model for RA. Our findings indicate that rolipram has a

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[4] Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; PDE IV, phosphodiesterase type IV; LNC, lymph node cell; PIP, proximal interphalangeal.

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highly significant ameliorative effect on arthritis. In addition, we demonstrate that combination therapy with rolipram plus anti-CD4 mAb gives even greater protection from arthritis, in a manner comparable with the previously reported therapeutic effect of combined anti-TNF-α/anti-CD4 treatment (28). Finally, we are able to report that the ameliorative effect of rolipram in this model is associated with profound changes in cytokine production, including reductions in the expression of TNF-α, IL-12, and IFN-γ and a corresponding increase in the expression of IL-10.

Materials and Methods

Induction and assessment of arthritis

Type II collagen was extracted from bovine articular cartilage as described (29). Male DBA/1 mice (8–10 wk of age) were immunized intradermally at the base of the tail with type II collagen (200 μg) emulsified in CFA (Difco, Detroit, MI). Clinical arthritis started to appear from day 14 after immunization, with a mean day of onset of 25 days. Drug/mAb treatment was started on the first day of clinical arthritis and was continued for 10 days. Assessment of arthritis over this 10-day period included monitoring of clinical scores using a clinical scoring system as follows: 0 = normal; 1 = slight swelling and/or erythema; 2 = pronounced edematous swelling. Each limb was graded in this way, giving a maximum possible score of 8 per mouse.

On day 10 of arthritis, mice were killed and the first paw to show clinical arthritis was decalcified, sectioned, and stained with hematoxylin and eosin. To determine whether a particular treatment had a protective effect on joint erosion, the proximal interphalangeal (PIP) joint of the middle digit was studied for the presence or absence of erosions, as defined previously (28). Erosions are detected in this joint in approximately 90 to 100% of untreated arthritic mice on day 10 of arthritis, and a significant reduction in this figure following treatment can be regarded as a beneficial therapeutic effect.

Serum levels of anti-collagen IgG on day 10 of arthritis were determined by direct ELISA, as described previously (10).

Therapeutic agents

Rolipram. Rolipram, 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone), was kindly provided by Schering AG (Berlin, Germany). Rolipram was dissolved in PBS containing cremophor (Sigma Chemical Co., Poole, U.K.) at a concentration of 10% (w/v). Rolipram was administered by i.p. injection at doses of 10, 5, 3, or 0.5 mg/kg body weight, twice daily. Control mice were given cremophor alone.

Anti-TNF-α mAb. TN3-19.12, a neutralizing hamster IgG1 mAb, was a gift from Dr. R. Schreiber in conjunction with Celltech, Slough, U.K. TN3-19.12 was administered by i.p. injection at a dose of 300 μg/mouse, once every third day. This dosing regimen had earlier been shown to be effective in reducing the severity of CIA (10).

Anti-CD4 mAb. Anti-CD4 consisted of a mixture (1:1) of two cell-depleting rat IgG2a mAbs, YTS 191.1.2 and YTA 3.1.2 (30–32). YTS 191.1.2 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, U.K.), and YTA 3.1.2 cells were kindly provided by Dr. Herman Waldmann (then at the University of Cambridge, U.K.). Both mAbs were prepared from ascites by protein G chromatography and injected by i.p. injection at a dose of 50 μg/mouse every third day.

TNF-α expression in situ

Arthritic mice were treated with rolipram (5 mg/kg twice daily) or vehicle alone for a period of 6 days, starting on the day of onset of clinical arthritis. Immunohistochemical analysis of TNF-α expression in frozen sections of joints from treated mice was carried out as previously described (33). TNF-α-positive cells were detected in the metatarsal joint (including intimal and subintimal layers) using a rat mAb (MP6-XT22; American Type Culture Collection (ATCC), Rockville, MD; courtesy of Dr. J. Abrams) and the ABC peroxidase system (Elite Kit, Vector, Peterborough, U.K.). Sections were developed with DAB (3,3'-diaminobenzidene) and counter-stained with Mayer's hematoxylin. The total number of cells in the joint was determined by counting the nuclei.

Th1/Th2 cytokine production in vitro

Arthritic mice were treated with rolipram (5 mg/kg, twice daily) or vehicle alone for 2 days, starting on the day of onset of arthritis. The mice were then killed, and their inguinal lymph nodes were removed and teased apart to make a single-cell suspension. The production of IFN-γ (Th1 cytokine) and IL-10 (Th2 cytokine) by lymph node cells (LNC) in response to stimulation with type II collagen (50 μg/ml) was assessed using a protocol previously described in detail (34). Briefly, LNC were cultured in 96 well plates in RPMI 1640, containing 10% FCS for 72 h. Culture supernatants were assayed for IFN-γ and IL-10 production by ELISA using the following mAbs: IL-10, 2A5 (capture), and SXC-1 (detect); IFN-γ, R4-6A2 (capture), and XG1.2 (detect). These Abs were obtained from the ATCC, courtesy of Dr. J. Abrams at DNAX (Palo Alto, CA).

Effect of rolipram on macrophage function in vitro

Bone marrow macrophages were derived from normal DBA/1 mice and cultured for 6 days with DMEM containing FCS (20%), 2-ME (50 μM), penicillin/streptomycin, and L cell (L-929) fibroblast-conditioned medium (15%). The differentiated cells were then rested for 2 days in fresh medium without conditioned medium. The cells were then primed with IFN-γ (100 U/ml) in the presence or absence of rolipram (dissolved in DMEM) and stimulated with 100 ng/ml of LPS B from *Escherichia coli* 055:B5 (Difco). Cells were incubated for 24 h, and supernatants were assayed by ELISA for the presence of TNF-α using XT3 (capture mAb; ATCC) and polyclonal rabbit anti-TNF-α (detect mAb; a gift from Dr. W. Buurman) and IL-12 p75 using gA5/5C3 (capture/detect mAbs, gifts from Dr. M. Gately, Hoffmann-La Roche, Nutley, NJ). Nitric oxide production was estimated from the concentration of nitrite in the culture supernatant using the Greiss reaction (35). The MTT assay was used to determine cell viability (36).

Results

Effect of rolipram on established CIA

To determine the effect of rolipram on the clinical course of CIA, the drug was administered in a range of concentrations (0.5, 3, 5, and 10 mg/kg) twice daily, starting on the day of onset of clinical arthritis. Treatment was continued for 10 days, and mice were monitored on a daily basis over this period. The results demonstrated a dose-dependent effect of rolipram on the clinical severity of arthritis, although with significant reductions compared with controls at all doses tested (Fig. 1). Little difference was observed between the 5 and 10 mg/kg doses; therefore, all further experiments were carried out at the 5 mg/kg dose. In terms of clinical disease, the therapeutic effect of rolipram was comparable with or greater than anti-TNF-α mAb.

At the end of the 10-day treatment period, mice were killed and PIP joints were analyzed for the presence of erosions (Fig. 2). Compared with control arthritic mice, there was a reduction of 21% in the proportion of joints showing erosions of cartilage and/or bone in the group of mice given rolipram at 5 mg/kg and a reduction of 49% in the mice given rolipram at 10 mg/kg (Table I). Erosions were reduced by 27% in the mice given anti-TNF-α mAb ($p < 0.02$), indicating that the protective effect of TNF-α blockade was comparable with rolipram at doses of 3 mg/kg or higher.

Mechanisms of action: rolipram inhibits the expression of TNF-α and IL-12

To confirm that treatment with rolipram leads to reduced expression of TNF-α at the site of disease activity, an immunohistochemical study was carried out to compare the number of TNF-α positive cells in the joints of rolipram treated versus control mice. Rolipram was administered at 5 mg/kg for 5 days, starting on day 1 of arthritis. Arthritic paws were snap frozen, cryosectioned, and analyzed for TNF-α expression on day 6 of arthritis. Day 6 was chosen for this study because we had earlier documented the presence of abundant TNF-α-positive cells in the joints of untreated mice with CIA at this time (33). In this study, it was found that the majority of TNF-α-expressing cells were macrophage-like in appearance, as reported previously (33). There was a reduction of 67% in the total number of cells expressing TNF-α in the joints of mice treated with rolipram compared with controls (Table II). In addition, the proportion of cells in the inflamed synovia that stained positively for TNF-α was reduced by 64% in the rolipram-treated mice, indicating that the reduction of TNF-α-positive cells

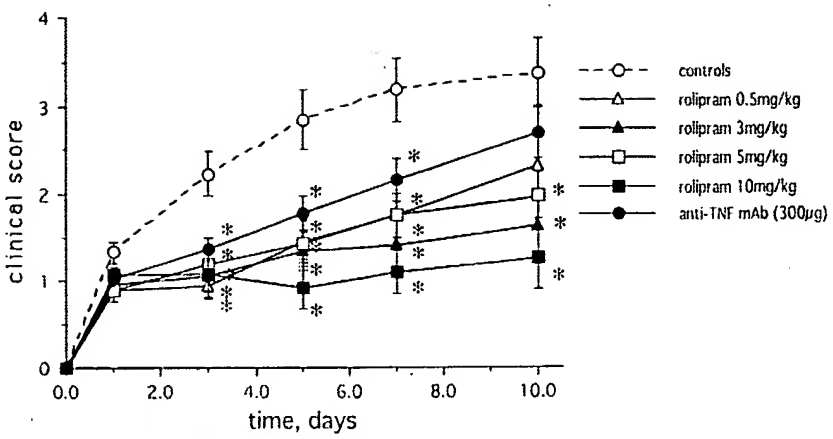

FIGURE 1. Therapeutic effect of rolipram in established CIA. Mice were treated with rolipram twice daily at 0.5 mg/kg (n = 10), 3 mg/kg (n = 16), 5 mg/kg (n = 16), or 10 mg/kg (n = 6), starting on day 1 of arthritis. Another group was given anti-TNF-α mAb (300 μg) on days 1, 4, and 7 (n = 16). Controls received vehicle alone (n = 16). An asterisk represents a significant difference ($p < 0.05$) between control and treated mice. Groups were compared using the Mann-Whitney U test.

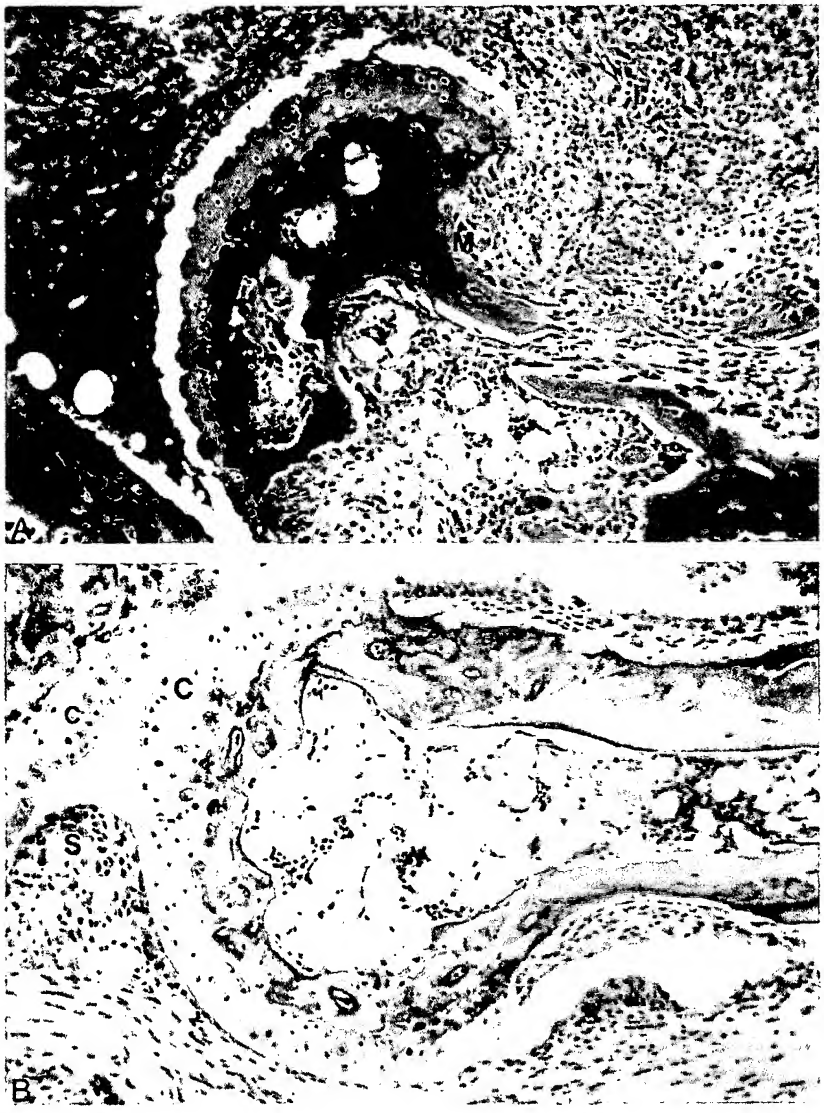

FIGURE 2. Saggital sections of PIP joints from mice on day 10 of arthritis. *A*, Control mouse showing pronounced inflammatory changes accompanied by marginal erosion and disruption of the articular cartilage (I, inflammation; M, marginal zone). *B*, Mouse treated with rolipram (5 mg/kg, twice daily) showing little evidence of inflammation and no destructive changes (C, cartilage; S, synovium). Stained with hematoxylin and eosin.

could be attributed, for the most part, to a reduction in TNF-α expression by cells in the joint rather than by reduced cellularity of the joint.

PDE IV inhibitors such as rolipram are known to exert a range of inhibitory effects on multiple cell types, including macrophages and lymphocytes. The reduction in TNF-α expression observed in our experiments could therefore have arisen from a direct inhibition of macrophage function and/or a change in the nature of the T cell response to type II collagen. To address the question of whether rolipram could directly inhibit the production of TNF-α by macrophages, bone marrow-derived macrophages were primed in vitro with IFN-γ in the presence or absence of rolipram, then

Table I. *Prevention of joint erosion by rolipram*[a]

Treatment	PIP Joints with Erosions (day 10)
Controls	25/28 (89%)
Rolipram, 0.5 mg/kg	7/9 (78%), NS
Rolipram, 3 mg/kg	8/13 (61%), $p < 0.05$
Rolipram, 5 mg/kg	17/25 (68%), $p = 0.05$
Rolipram, 10 mg/kg	2/5 (40%), $p < 0.01$
Anti-TNF-α mAb (300 μg)	18/29 (62%), $p < 0.02$

[a] Arthritic mice were treated for 10 days, and paws were processed for hematoxylin and eosin staining followed by histologic analysis of joint erosion. p values were obtained using the χ^2 test or the Fisher's exact test, where appropriate.

Table II. *Immunohistochemical analysis of TNF-α expression in frozen sections of joints of rolipram-treated mice*[a]

Treatment	TNF-α-Positive Cells per Metatarsal Joint (mean ± SE)	Ratio of TNF-α-Positive Cells:Total Cells
Controls ($n = 7$)	55 (± 6)	1:20
Rolipram ($n = 7$)	18 (± 4) $p < 0.05$	1:55

[a] Treatment with rolipram (5 mg/kg, twice daily) leads to a reduction in the number of TNF-α-positive cells in the metatarsal joint. Treatment was started on day 1 of arthritis and continued until day 6. TNF-α expression was determined using anti-TNF-α mAb (XT22) and an avidin/biotin detection system.

costimulated 24 h later with LPS. Supernatants were then assayed for TNF-α, IL-12, and nitric oxide. The results clearly showed that rolipram directly inhibits the secretion of TNF-α and IL-12 at doses that do not adversely affect cell viability (Fig. 3). Thus, the presence of rolipram at a concentration of 1 mM caused a 20-fold reduction in the production of TNF-α and an 8-fold reduction in the production of IL-12. IL-10 was not detected in culture supernatants of control or rolipram-treated macrophages, but this is probably due to the fact that the cells were costimulated with IFN-γ, a potent inhibitor of IL-10 expression (37). Nitric oxide production was not affected at the doses tested.

Rolipram down-regulates Th1 activity

The finding that rolipram inhibits the production of IL-12 by macrophages raises the possibility that in vivo administration of the drug would lead to down-regulation of the Th1 response to type II collagen in CIA. To test this hypothesis, mice with CIA were treated with rolipram (5 mg/kg, twice daily) from day 1 to day 3 of arthritis. On day 3, the mice were killed and the production of IFN-γ (Th1 cytokine) and IL-10 (Th2 cytokine) by cultured LNC was assessed using an established protocol (34). The results showed that IFN-γ production by type II collagen-stimulated LNC from rolipram-treated mice was decreased 8-fold relative to control arthritic mice, whereas the production of IL-10 was increased 11-fold (Fig. 4). It was concluded that treatment with rolipram leads to a down-regulation of Th1 activity and a corresponding up-regulation of the Th2 cytokine, IL-10. Of interest was the observation that collagen stimulation of LNC from both control and rolipram-treated mice caused a dramatic increase in IFN-γ production, which could be blocked by anti-CD4 mAb. In contrast, IL-10 production was not increased by coculture with collagen or blocked by anti-CD4 mAb, indicating that the production of IFN-γ, but not IL-10, was dependent on Ag-specific $CD4^+$ T cells.

Despite the clear shift from a Th1 to a Th2 cytokine response following treatment with rolipram, no differences were observed between treated and control mice with respect to the levels of type II collagen-specific IgG1, IgG2a, or total IgG on day 10 of arthritis

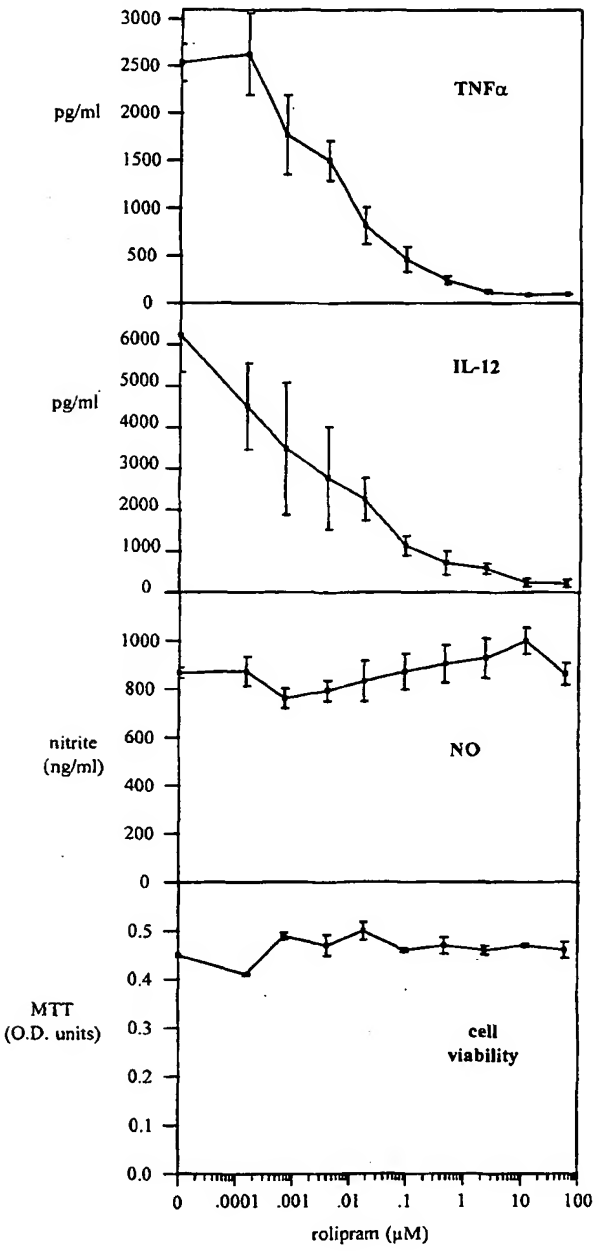

FIGURE 3. Dose-dependent suppression of macrophage-derived TNF-α and IL-12, but not nitric oxide, by rolipram. Bone marrow-derived macrophages were primed with IFN-γ, then stimulated with LPS. Rolipram was added with the IFN-γ. TNF-α and IL-12 levels were measured by ELISA, and nitric oxide production was estimated from the levels of nitrite (measured by the Greiss reaction). Cell viability was determined using the MTT assay. The results shown are the means (± SEM) of three separate cultures.

(data not shown). However, given the relatively long half-life of IgG Abs, it is unlikely that any significant qualitative or quantitative changes in the anti-collagen IgG response would be detected over this brief 10-day treatment period.

Combination therapy

Thus far we had demonstrated that rolipram has inhibitory effects on TNF-α production and Th1 activity, although it was not possible from the above studies to determine which of these two

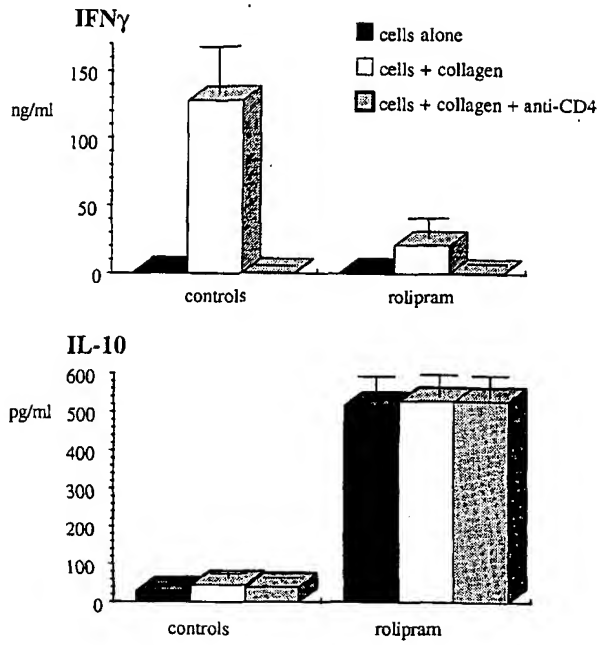

FIGURE 4. IFN-γ and IL-10 production by draining LNC from rolipram-treated and control arthritic mice. Cells were cultured for 72 h in medium alone, with type II collagen, or with type II collagen plus anti-CD4 mAb. Rolipram (5 mg/kg, twice daily) was administered from day 1 to day 3 of arthritis ($n = 5$). Controls received vehicle alone ($n = 7$). Cultures were carried out in triplicate.

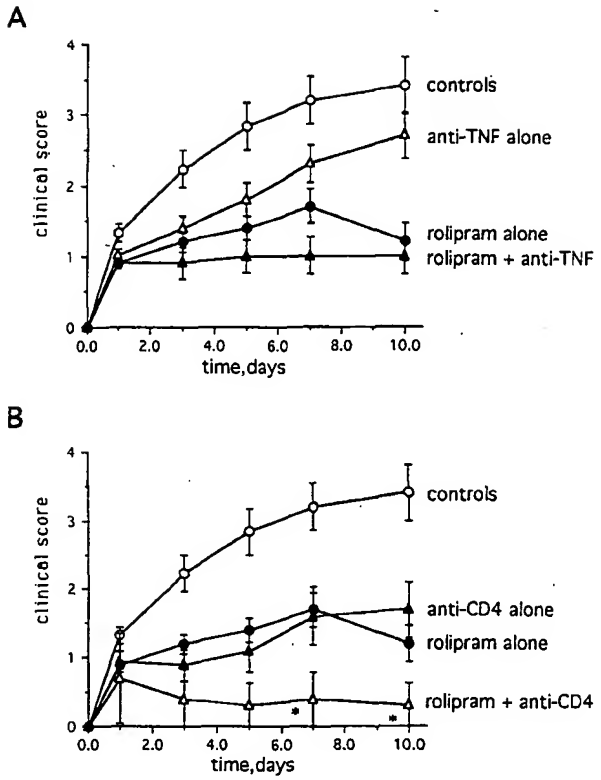

FIGURE 5. Combination therapy. Treatment of mice with rolipram (5 mg/kg, twice daily) plus anti-TNF-α mAb (300 μg/mouse, every 3 days; *A*) or anti-CD4 mAb (50 μg/mouse, every three days; *B*). There were 12 mice per treatment group. Significant differences between the combined treatment groups and the single treatment groups are denoted by an asterisk.

mechanisms, if either, was the more important in terms of ameliorating arthritis. Previously we had demonstrated a synergistic therapeutic effect between anti-TNF-α mAb and anti-CD4 mAb (28). We now tested the effect of rolipram in combination with either anti-TNF-α mAb or anti-CD4 mAb to establish whether the therapeutic effects of rolipram were complimentary to, or overlapping with, either of these two reagents.

In combination with anti-TNF-α mAb, rolipram had a slightly greater effect than either anti-TNF-α alone or rolipram alone, but the differences were not significant. It was concluded that this form of combination therapy did not provide an additive effect (Fig. 5*A*). An additive effect, for the purposes of this study, was defined as a significant decrease in disease severity in the combined treatment group versus either of the two treatments alone.

Combined treatment with rolipram and anti-CD4 mAb caused almost complete cessation of ongoing disease, with significantly reduced severity of disease relative to rolipram alone or anti-CD4 alone (Fig. 5*B*). We interpreted this finding as evidence of an additive effect between rolipram and anti-CD4, suggesting that the benefits derived from the two reagents were generated by different pathways. In contrast, the lack of additive effect between rolipram and anti-TNF-α suggests overlapping mechanisms of action. It was concluded from this part of the study that one of the major mechanisms of action of rolipram in CIA is via suppression of TNF-α activity. However, the fact that rolipram was more effective than anti-TNF-α mAb suggests that its inhibitory effect on Th1 activity represents an important additional factor in the amelioration of arthritis.

Discussion

From earlier studies, it was hypothesized that PDE IV inhibitors possess potent anti-inflammatory properties, based on their ability to down-regulate the activity of a number of leucocyte populations, including macrophages and lymphocytes (19, 25). The aim of this study was to assess the efficacy in vivo of rolipram, a selective PDE IV inhibitor, in murine CIA, as a model for human RA. In an initial experiment, it was found that rolipram, administered after the onset of visible arthritis, had a dose-dependent therapeutic effect both in terms of ameliorating clinical disease and in preventing erosion of cartilage and bone. The efficacy of rolipram was comparable with, or greater than, that of anti-TNF-α Ab treatment.

Having demonstrated that rolipram has a beneficial therapeutic effect in CIA, we then set out to analyze the mechanisms involved in the amelioration of disease. Immunohistochemical analysis of the joints of arthritic mice demonstrated that rolipram suppresses TNF-α expression, as judged by the reduction in the proportion of TNF-α-positive cells detected in rolipram-treated mice. However, from this study it was not possible to confirm that rolipram is capable of directly suppressing TNF-α production by macrophages or whether the inhibitory effect observed in situ is an indirect consequence of, for example, a reduced level of T cell activity. To address this question, mature homogenous populations of cultured bone marrow-derived macrophages were stimulated with IFN-γ/LPS and treated concurrently with rolipram. The results confirmed that rolipram, as postulated (20), does indeed directly suppress TNF-α production by macrophages. An additional finding that we regard as highly significant was the ability of rolipram to suppress the production of IL-12, a cytokine that is postulated to play a pivotal role in stimulating the expression of IFN-γ and influencing the differentiation of T cells toward a Th1 phenotype. The inhibitory effects of rolipram on cytokine expression were selective, as

nitric oxide production was unaffected at the doses tested. It is also worth pointing out that rolipram did not affect cell viability even up to a concentration of 1 M (data not shown).

In view of the ability of rolipram to inhibit the production of IL-12 in vitro, we addressed the question of whether in vivo administration of the drug would result in a blunting of the Th1 response to type II collagen. Previous findings by our group had established that early CIA was associated with a potent Th1 response, with high levels of IFN-γ production and negligible production of IL-4 and IL-10 (34). We therefore determined the effect of in vivo administration of rolipram on Th1/Th2 cytokine production by cultured LNC from mice with arthritis. It was demonstrated that IFN-γ production in rolipram-treated mice was dramatically reduced, while the production of IL-10 was correspondingly increased. This finding was particularly striking in view of the short treatment period (2 days) and also because of the fact that treatment was not started until after the onset of arthritis. Rolipram was therefore effective in deviating an established immune response that was already fully committed toward the Th1 end of the spectrum. This finding strongly supports the concept that PDE IV inhibitors may be effective in human autoimmune diseases (e.g., RA) in which Th1 responses are thought to predominate. Conversely, a cautious approach should be taken with regard to the potential use of PDE IV inhibitors in diseases in which Th2 responses play significant roles (e.g., asthma). In fact, PDE IV inhibitors have already been used in clinical trials for asthma, so far without clear success.

Earlier studies by our group had established a synergistic therapeutic effect between anti-CD4 and anti-TNF mAb (28). Given the dual role of rolipram in suppressing TNF-α production and down-regulating Th1 activity, we tested the effect of rolipram combined with either anti-TNF-α or anti-CD4. The reasons for carrying out this study were (1) to provide clues as to the principal mechanism of action of rolipram in CIA and (2) to identify a form of combination therapy that may be applicable to human RA. The results showed that combined treatment with rolipram plus anti-TNF-α mAb did not have a significant additive effect. In contrast, rolipram plus anti-CD4 mAb showed a clear additive effect. This would suggest that the effects of rolipram overlap with those of anti-TNF-α but not with anti-CD4. From these findings, we could infer that one of the major mechanisms of action of rolipram in CIA is the suppression of TNF-α activity. However, there was a trend toward greater efficacy of rolipram compared with anti-TNF-α mAb, which, although not statistically significant, suggests that rolipram has pleiotropic effects. Indeed, we have provided evidence that rolipram affects the activation status of T cells (Fig. 4). Alternatively, we have found that there is a marked additive effect between anti-TNF-α mAb and anti-IL-12 mAb[5] and rolipram, by virtue of its ability to inhibit both TNF-α and IL-12 production (Fig. 3), appears to be mimicking this synergy.

The profound amelioration of arthritis observed in mice treated with a combination of rolipram and anti-CD4 mAb has potential implications for human therapy as anti-CD4 treatment alone has been shown, in placebo-controlled trials at least, to be ineffective in RA (38). If the results of this study are valid for human disease, then treatment of RA patients with anti-CD4 mAb together with a PDE IV inhibitor would be highly effective.

In conclusion, we have demonstrated that rolipram is capable of ameliorating CIA, and that PDE IV inhibitors may be useful drugs in the future therapy of RA provided that their side effects (such as emesis) can be managed. Increasingly selective PDE IV inhibitors have been or are being developed, making them potential therapeutic agents for RA.

Note added in proof. Since submission of this manuscript, similar findings have been reported in the rat model of collagen-induced arthritis. (Nyman, U., A., Mussener, E. Larsson, and J. Lorentzen. 1997. Amelioration of collagen IL-induced arthritis in rats by the type IV phosphodiesterase inhibitor Rolipram. *1997 Clin. Exp. Immunol. 108:415.)*

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References

1. Brennan, F. M., D. Chantry, A. Jackson, R. Maini, and M. Feldmann. 1989. Inhibitory effect of TNFα antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet 2:244.*
2. Chu, C. Q., M. Field, M. Feldmann, and R. N. Maini. 1991. Localization of tumor necrosis factor alpha in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis. *Arthritis Rheum. 34:1125.*
3. Haworth, C., F. M. Brennan, D. Chantry, M. Turner, R. N. Maini, and M. Feldmann. 1991. Expression of granulocyte-macrophage colony-stimulating factor in rheumatoid arthritis: regulation by tumor necrosis factor-α. *Eur. J. Immunol. 21:2575.*
4. Dayer, J. M., B. Beutler, and A. Cerami. 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. *J. Exp. Med. 162:2163.*
5. Gamble, J. R., J. M. Harlan, S. J. Klebanoff, and M. A. Vadas. 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA 82:8667.*
6. Cavender, D., Y. Saegusa, and M. Ziff. 1987. Stimulation of endothelial cell binding of lymphocytes by tumor necrosis factor. *J. Immunol. 139:1855.*
7. Thorbecke, G. J., R. Shah, C. H. Leu, A. P. Kuruvilla, A. M. Hardison, and M. A. Palladino. 1992. Involvement of endogenous tumor necrosis factor α and transforming growth factor β during induction of collagen type II arthritis in mice. *Proc. Natl. Acad. Sci. USA 89:7375.*
8. Cooper, W. O., R. A. Fava, C. A. Gates, M. A. Cremer, and A. S. Townes. 1992. Acceleration of onset of collagen-induced arthritis by intra-articular injection of tumour necrosis factor or transforming growth factor-beta. *Clin. Exp. Immunol. 89:244.*
9. Brahn, E., D. J. Peacock, M. L. Banquerigo, and D. Y. Liu. 1992. Effects of tumor necrosis factor alpha (TNF-α) on collagen arthritis. *Lymphokine Cytokine Res. 11:253.*
10. Williams, R. O., M. Feldmann, and R. N. Maini. 1992. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc. Natl. Acad. Sci. USA 89:9784.*
11. Piguet, P. F., G. E. Grau, C. Vesin, H. Loetscher, R. Gentz, and W. Lesslauer. 1992. Evolution of collagen arthritis in mice is arrested by treatment with anti-tumour necrosis factor (TNF) antibody or a recombinant soluble TNF receptor. *Immunology 77:510.*
12. Elliott, M. J., R. N. Maini, M. Feldmann, A. Long-Fox, P. Charles, P. Katsikis, F. M. Brennan, J. Walker, H. Bijl, J. Ghrayeb, and J. N. Woody. 1993. Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumour necrosis factor α. *Arthritis Rheum. 36:1681.*
13. Waldmann, T. A. 1991. Monoclonal antibodies in diagnosis and therapy. *Science 252:1657.*
14. Wachtel, H. 1982. Characteristic behavioural alterations in rats induced by rolipram and other selective adenosine cyclic 3',5'-monophosphate phosphodiesterase inhibitors. *Psychopharmacology 77:309.*
15. Wachtel, H. 1990. The second-messenger dysbalance hypothesis of affective disorders. *Pharmacopsychiatry 23:27.*
16. Wachtel, H. 1983. Neurotropic effects of the optical isomers of the selective adenosine cyclic 3',5'-monophosphate phosphodiesterase inhibitor rolipram in rats in vivo. *J. Pharm. Pharmacol. 35:440.*
17. Krause, W., G. Kuhne, and H. Matthes. 1989. Pharmacokinetics of the antidepressant rolipram in healthy volunteers. *Xenobiotica 19:683.*
18. Nicholson, C. D., R. A. Challiss, and M. Shahid. 1991. Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci. 12:19.*
19. Sekut, L., D. Yarnall, S. A. Stimpson, L. S. Noel, R. Bateman-Fite, R. L. Clark, J. A. Menius, Jr., and K. M. Connolly. 1995. Anti-inflammatory activity of phosphodiesterase (PDE)-IV inhibitors in acute and chronic models of inflammation. *Clin. Exp. Immunol. 100:126.*
20. Semmler, J., H. Wachtel, and S. Endres. 1993. The specific type IV phosphodiesterase inhibitor rolipram suppresses tumor necrosis factor-alpha production by human mononuclear cells. *Int. J. Immunopharmacol. 15:409.*
21. McLaughlin, M. M., L. B. Cieslinski, M. Burman, T. J. Torphy, and G. P. Livi. 1993. A low-Km, rolipram-sensitive, cAMP-specific phosphodiesterase from human brain: cloning and expression of cDNA, biochemical characterization of recombinant protein, and tissue distribution of mRNA. *J. Biol. Chem. 268:6470.*

[5] D. M. Butler, A.-M. Malfait, R. N. Maini, F. M. Brennan and M. Feldmann. Anti-IL-12 and anti-TNF blockade synergistically suppresses the progression of murine collagen induced arthritis. *Submitted for publication.*

22. Turner, N. C., L. J. Wood, F. M. Burns, T. Gueremy, and J. E. Souness. 1993. The effect of cyclic AMP and cyclic GMP phosphodiesterase inhibitors on the superoxide burst of guinea-pig peritoneal macrophages. *Br. J. Pharmacol. 108:876.*
23. Suttorp, N., U. Weber, T. Welsch, and C. Schudt. 1993. Role of phosphodiesterases in the regulation of endothelial permeability in vitro. *J. Clin. Invest. 91:1421.*
24. Bulut, V., A. Severn, and F. Y. Liew. 1993. Nitric oxide production by murine macrophages is inhibited by prolonged elevation of cyclic AMP. *Biochem. Biophys. Res. Commun. 195:1134.*
25. Torphy, T. J. and B. J. Undem. 1991. Phosphodiesterase inhibitors: new opportunities for the treatment of asthma. *Thorax 46:512.*
26. Sommer, N., P. A. Loschmann, G. H. Northoff, M. Weller, A. Steinbrecher, R. Lichtenfels, R. Meyermann, A. Riethmuller, and A. Fontana. 1995. The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. *Nat. Med. 1:244.*
27. Genain, C. P., T. Roberts, R. L. Davis, M. H. Nguyen, A. Uccelli, D. Faulds, Y. Li, J. Hedgpeth, and S. L. Hauser. 1995. Prevention of autoimmune demyelination in non-human primates by a cAMP-specific phosphodiesterase inhibitor. *Proc. Natl. Acad. Sci. USA 92:3601.*
28. Williams, R. O., L. J. Mason, M. Feldmann, and R. N. Maini. 1994. Synergy between anti-CD4 and anti-TNF in the amelioration of established collagen-induced arthritis. *Proc. Natl. Acad. Sci. USA 91:2762.*
29. Miller, E. J. 1972. Structural studies on cartilage collagen employing limited cleavage and solubilization with pepsin. *Biochemistry 11:4903.*
30. Galfre, G., C. Milstein, and B. Wright. 1979. Rat x rat hybrid myelomas and a monoclonal anti-Fd portion of mouse IgG. *Nature 277:131.*
31. Cobbold, S. P., A. Jayasuriya, A. Nash, T. D. Prospero, and H. Waldmann. 1984. Therapy with monoclonal antibodies by elimination of T-cell subsets in vivo. *Nature 312:548.*
32. Qin, S., S. Cobbold, H. Tighe, R. Benjamin, and H. Waldmann. 1987. CD4 monoclonal antibody pairs for immunosuppression and tolerance induction. *Eur. J. Immunol. 17:1159.*
33. Marinova-Mutafchieva, L., R. O. Williams, L. J. Mason, C. Mauri, M. Feldmann, and R. N. Maini. 1997. Dynamics of proinflammatory cytokine expression in the joints of mice with collagen-induced arthritis (CIA). *Clin. Exp. Immunol. 107:507.*
34. Mauri, C., R. O. Williams, M. Walmsley, and M. Feldmann. 1996. Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. *Eur. J. Immunol 26:1511.*
35. Roach, T. I., A. F. Kiderlen, and J. M. Blackwell. 1991. Role of inorganic nitrogen oxides and tumor necrosis factor alpha in killing *Leishmania donovani* amastigotes in gamma interferon-lipopolysaccharide-activated macrophages from *Lshs* and *Lshr* congenic mouse strains. *Infect. Immun. 59:3935.*
36. Gerlier, D., and N. Thomasset. 1986. Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods 94:57.*
37. Mosmann, T. R. 1994. Properties and functions of interleukin-10. *Adv. Immunol. 56:1.*
38. van der Lubbe, P. A., B. A. C. Dijkmans, H. M. Markusse, U. Nassander, and F. C. Breedfeld. 1994. Lack of clinical effect of CD4 monoclonal antibody therapy in early rheumatoid arthritis: a placebo-controlled trial. *Arthritis Rheum. 37(Suppl.):S294 (Abstr.).*

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Dextran Sulfate Sodium-Induced Colonic Histopathology, but not Altered Epithelial Ion Transport, Is Reduced by Inhibition of Phosphodiesterase Activity

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Inhibition of phosphodiesterase (PDE) activity is beneficial in models of arthritis and airway inflammation. Here we assessed the ability of PDE inhibitors to modulate colitis by exposing mice to 4% (w/v) dextran sulfate sodium (DSS) drinking water for 5 days with or without rolipram, an inhibitor of PDE type 4, or the nonselective PDE inhibitor, pentoxifylline (both at 5 mg/kg, i.p., twice daily). Controls received saline, vehicle, or drug only. Colonic histology, myeloperoxidase (MPO) and tumor necrosis factor-α (TNF-α) levels, and epithelial ion transport (baseline and stimulated by electrical nerve stimulation, carbachol, and forskolin) were examined. DSS-treated mice displayed a variable diarrhea, significant histopathology in the mid-distal colon, elevated MPO activity, and reduced (>50%) responses to all three pro-secretory stimuli. Treatment with rolipram, and to a lesser extent pentoxifylline, significantly reduced the severity of the colonic histopathology and MPO levels. Neither PDE inhibitor had any affect on the diminished ion transport events caused by DSS-induced colitis. However, although stimulated ion transport events were still reduced 3 days after DSS treatment, colonic segments from DSS + rolipram-treated mice displayed enhanced recovery in their secretory responsiveness, particularly to carbachol. These findings indicate that specific PDE4 inhibition can significantly reduce the tissue damage that accompanies colitis and enhance recovery of normal colonic function. ***(Am J Pathol 2000, 156:2169–2177)***

Generally elevations in cyclic adenosine monophosphate (cAMP) levels in immune cells have been found to be immunosuppressive.[1] For instance, increased macrophage cAMP has been associated with a reduced capacity to produce TNF-α on stimulation with bacterial endotoxin.[2] cAMP is hydrolyzed to the inactive 5′AMP form by members of a superfamily of enzymes, the phosphodiesterases (PDE), of which type 4 (PDE4) is the predominant form in immune cells.[1,3,4] Thus, inhibition of PDE activity that would result in maintained elevation of cAMP levels in immune cells presents itself as a potential anti-inflammatory or immunosuppressive strategy.[5,6]

In fact, numerous *in vitro* studies have documented that nonselective PDE, or specific PDE4 inhibitors, down-regulate the activity of most types of immune cells, including T-cells, macrophages, neutrophils, and mast cells.[1,3] Similarly, PDE inhibitors have been found to be beneficial in animal models of airway or joint inflammation.[7,8] In the latter scenario, it was suggested that the amelioration of collagen-induced arthritis in mice by the PDE4 inhibitor, rolipram (ROL), was because of suppression of TNF-α production. In this context, it is noteworthy that neutralizing TNF-α antibodies are an effective short-term treatment for a cohort of steroid-resistant patients with Crohn's disease.[9] However, the ability of inhibitors of PDE activity to alleviate either the functional abnormalities or histopathology associated with enteric inflammation has not been extensively examined.

Oral administration of dextran sulfate sodium (DSS) to rodents results in overt inflammation in the mid-distal colon that is somewhat reminiscent of human inflammatory bowel disease. Exposure to DSS leads to a time- and dose-dependent drop in body weight, a variable watery/bloody diarrhea, and can result in rectal prolapse and fatality.[10,11] A number of approaches have been used to reduce the severity of DSS-induced histopathology in the colon, such as treatment with ICAM-1 anti-sense oligonucleotides,[12] recombinant IL-10,[13] inhibition of 5-lipoxygenase or neutrophil activity,[14,15] and neutralization of TNF-α.[16] However, considerably fewer studies have ex-

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Table 1. Macroscopic Assessment of Animals

	Control	4% DSS (5 days)	DSS + ROL	DSS + PTX
Clinical signs (% of mice)				
Wet peri-anal area/fluid-filled lumen	0	56	50	16
Semi-solid fecal pellet	0	39	100	81
Hyperemic colon/occult blood/bloody diarrhea	0	73	50	97
Colon length (mm)	87 ± 2	67 ± 2*	77 ± 3†	78 ± 3†
Change in body weight (g/5 days)	0.26 ± 0.12	−1.16 ± 0.36*	−2.75 ± 7†	−1.02 ± 0.31*

Mean ± SEM; n = 9 to 20 mice per group.
*, $P < 0.05$ compared to control and †, $P < 0.05$ compared to control and DSS only; drugs were administered intraperitoneally twice a day at 5 mg/kg.

amined changes in colonic physiology as a consequence of exposure to DSS.

The present study was designed to compare the capacities of the nonselective PDE inhibitor, pentoxifylline (PTX), and the PDE4 inhibitor, ROL, to affect gut inflammation as judged by both structure and epithelial ion transport in the DSS model of murine colitis. The data show that concomitant twice-daily treatment with ROL, and to a lesser extent PTX, significantly reduced the severity of the colonic histopathology induced by a 5-day course of DSS drinking water. However, the disrupted epithelial ion transport apparent at the end of the DSS-treatment period was not affected by either inhibitor of PDE activity.

Materials and Methods

Animals and Experimental Treatment

Male BALB/c mice (6 to 8 weeks old) were purchased from Harlan Animal Suppliers (Indianapolis, IN) and were housed under conventional conditions with free access to animal chow and water. For the induction of colitis, normal drinking water was replaced with a 4% (w/v) solution of DSS (molecular weight, 40 kd; ICN Biomedicals Inc., Aurora, OH) for 5 days.[17] PTX or ROL (both from Sigma Chemical Co., St. Louis, MO) were administered twice daily, intraperitoneally (i.p.) at a dose of 5 mg/kg body weight in 200 μl of phosphate-buffered saline (PBS) beginning on the day of DSS exposure.[7,8,18,19] Pilot studies examined the effect of a single daily injection of ROL at the same dose. Time-matched controls consisted of naïve mice, mice administered DSS, ROL, or PTX only, and those injected with ethanol (10 μl in 190 μl of PBS) only, the vehicle for ROL. All experiments were approved by the Animal Care Committee at McMaster University.

Macroscopic Assessment

Animals were weighed and their behavior observed daily. On day 5, water intake was recorded (expressed as ml/day/mouse), mice were sacrificed by cervical dislocation, and the entire colon (from ileocecal junction to the anus) was excised. Colon length was measured and observations regarding ulceration, vascularization, and stool consistency were recorded (Table 1). Previous studies have shown colonic shortening during colitis,[10] and so the colon was divided based on total length: the mid-distal portion (ie, 30 to 60% region) was used for electrophysiology studies; the adjacent distal 10% was fixed for histological examination; and the remainder of the tissue was snap-frozen in liquid N_2 before processing for myeloperoxidase (MPO) activity or TNF-α levels.

Histology

Tissue was fixed in 10% neutral buffered formalin, dehydrated, and wax-embedded. Five-μm sections were collected on coded slides, stained with hematoxylin and eosin, and scored in a blinded fashion by two investigators. Histological damage was scored using the criteria of Appleyard and Wallace[20] which considers loss of mucosal architecture, cellular infiltration, muscle thickening, crypt abscess formation, and goblet cell depletion (maximum score = 11).

MPO Activity

MPO activity was determined following a published protocol.[21] Briefly, tissue samples were weighed and suspended in 50 mmol/L potassium phosphate buffer (pH 6.0) containing 5 mg/ml hexadecyltrimethylammonium bromide (Sigma Chemical Co.) at a ratio of 50 mg tissue to 1 ml of buffer. Tissues were homogenized by a polytron tissue homogenizer for 15 seconds, and 1 ml was decanted into sterile Eppendorf tubes and centrifuged at 12,000 rpm for 15 minutes. Using a microtiter plate scanner, 200 μl of the reaction mixture (containing 16.7 mg of *o*-dianisidine (Sigma Chemical Co.), 90 ml of distilled H_2O, 10 ml of potassium-phosphate buffer, and 50 μl of 1% H_2O_2) was added to each well containing 7 μl of sample in a standard 96-well plate and three absorbance readings at 30-second intervals at 450 nm were recorded. MPO activity was measured in units/mg tissue, where one unit of MPO was defined as the amount needed to degrade 1 μmol of H_2O_2 per minute at room temperature.

Functional Studies

Epithelial ion transport was examined in colonic segments (approximately mid-distal colon) mounted in Ussing chambers.[22] Tissues (surface area = 0.6 cm^2) were bathed in 10 ml of warm (37°C), oxygenated Krebs buffer (pH 7.35 ± 0.02). The spontaneous potential dif-

ference was maintained at 0 mV by an automated voltage clamp (WPI, Mississauga, Ontario, Canada), and the short-circuit current (Isc in $\mu A/cm^2$) was continuously measured as an indicator of net active ion transport. At 15-minute intervals, tissue conductance (G; indicates barrier to passive ion flow) was calculated from Isc and potential difference values.

Stimulated ion transport events were sequentially evoked by: 1) electrical transmural stimulation (10 Hz, 10 mA, 0.5 ms for a total time of 5 seconds); 2) addition of the cholinergic agonist, carbachol (10^{-4} mol/L; Sigma Chemical Co.) to the serosal buffer; and 3) addition of the adenylate cyclase-activating agent, forskolin (10^{-5} mol/L; Sigma Chemical Co.) to the serosal buffer.[23] In all instances, the effect of the treatment was recorded as the maximum change in Isc (ie, ΔIsc) to occur within 5 minutes.

Cytokine Production

At the end of the DSS-treatment period, blood was collected for determination of serum TNF-α levels by enzyme-linked immunosorbent assay using paired antibodies from PharMingen Inc. (detection limit 32 pg/ml; Mississauga, Canada). Portions of terminal colon were homogenized in PBS containing 2 mmol/L of phenylmethyl sulfonyl fluoride (Sigma Chemical Co.) and tissue levels of TNF-α were measured. All cytokine determinations were performed in duplicate serial dilutions.

In Vitro *Analysis of DSS Epithelial Toxicity*

The murine IEC-4.1 epithelial cell line was seeded in 12-well sterile plates (10^6 cells/well) and grown for 24 hours under standard culture conditions[24] and then exposed to 1% or 2% DSS ± ROL (10^{-6} mol/L). Twenty hours later the enterocytes were retrieved by addition of trypsin/ethylenediaminetetraacetic acid and cell viability determined using the trypan blue (0.04% w/v) dye exclusion technique. In another set of experiments, enterocytes were seeded into 96-well plates (3.5×10^4 cells/well) and 24 hours later were exposed to 1% or 2% DSS ± ROL for 20 hours. Subsequently each well was pulsed with 50 μg of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co.) for 5.5 hours followed by the addition of 50 μl of 10% (v/v) Triton-X 100/0.5 mol/L HCl and a 24-hour incubation in the dark. Optical density was measured at 540 nm.[25]

Recovery Experiments

In a final series of experiments, mice were exposed to 4% DSS for 5 days with or without daily ROL (5 mg/kg, twice daily). Subsequently, the animals received normal drinking water for 3 days (ROL was not administered during this period), and were then autopsied and the colonic form and function were assessed.

Analysis and Data Presentation

All data are expressed as means ± SEM (SEM), where *n* refers to the number of mice in each experiment. Data were compared using one-way analysis of variance (WINKS software by Texsoft, Cedarhill, TX) and $P < 0.05$ was accepted as the level of statistically significant difference compared to time-matched controls.

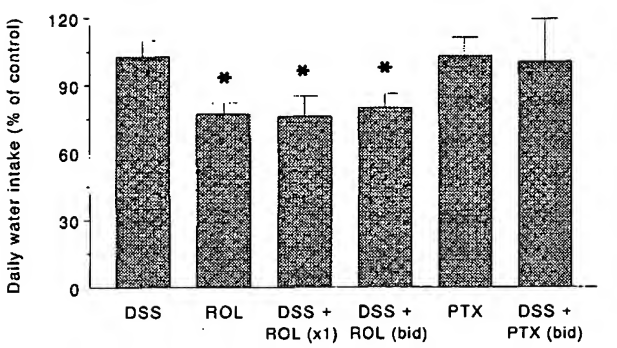

Figure 1. Bar chart showing average daily DSS water intake as a percentage of normal water intake in time-matched control mice. DSS, 4% (w/v) for 5 days; ROL, given i.p. at 5 mg/kg (ie, 100 μg/mouse) once or twice daily (twice daily); PTX, given i.p. at 5 mg/kg (ie, 100 μg/mouse) twice daily. Mean values of 3 to 5 separate experiments with 3 to 4 mice per group in each experiment; means ± SEM; *, $P < 0.05$ compared to control.

Results

Colons from mice treated with PBS, ethanol, ROL, or PTX only ($n = 3$ per group) were not significantly different for any parameter examined, and so PBS-injected mice were subsequently used as time-matched controls throughout the study. Mice treated with ROL, independent of DSS exposure, displayed a hind-end paralysis within 5 minutes of injection that lasted 10 to 20 minutes and thereafter the mice had normal ambulatory behavior. This paralysis is consistent with effects on neuronal PDEs. This effect was not observed in ethanol- or PTX-treated mice. Addition of DSS (4% w/v) to the water did not significantly alter animal daily water intake. In five separate experiments mice treated with ROL, once or twice daily, consumed less DSS/water (range, 4 to 28% reduction) than controls, although PTX therapy did not affect daily water intake (Figure 1). However, the beneficial effect of ROL (twice daily) described below is unlikely to be because of the reduced DSS intake because mice receiving ROL once a day developed a colitis virtually indistinguishable from that in the DSS-positive control group, despite their reduced DSS water intake (ie, histopathology damage scores were not significantly different when DSS and DSS + ROL, once a day, were compared).

Macroscopic Assessment

After 5 days of *ad libitum* exposure to 4% DSS many of the mice displayed clinical and macroscopic signs of inflammation and/or gut dysfunction. The distal two-thirds of the colon of DSS-treated mice was consistently devoid of contents, whereas the colon of DSS + ROL-treated mice typically contained loosely formed stool. Table 1 shows that all animals receiving DSS, independent of PDE treatment displayed some signs of diarrhea or perturbed water balance; however, luminal fluid content was not quan-

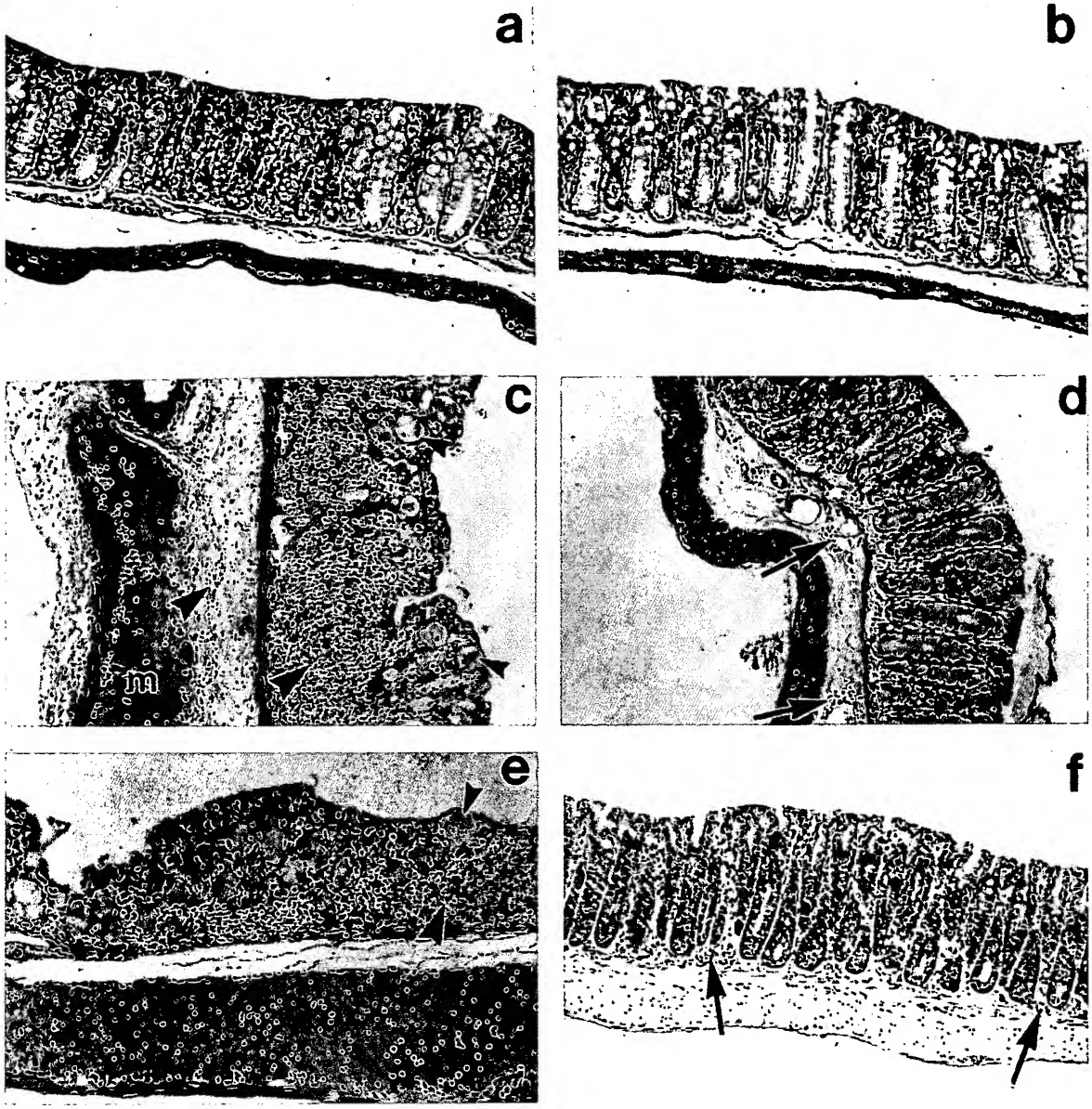

Figure 2. Representative photomicrographs of colonic segments from mice given regular water, ie, control (**a**); ROL only, twice daily (**b**); DSS only (**c**); DSS + ROL, twice daily (**d**); DSS + PTX, twice daily (**e**); and mice treated with DSS + ROL, twice daily for 5 days and then allowed to recover for an additional 3 days (**f**). m, muscle; **small arrowheads** denote edema; **large arrowheads** and **arrows** indicate inflammatory infiltrate. See Figure 1 legend for abbreviations and treatment regimen.

tified. Typical of other colitidies, the DSS-treated animals had a significantly shortened colon, which was partially prevented by ROL and PTX (Table 1). In addition, DSS treatment resulted in a significant drop in body weight that was not abrogated by PTX and was exaggerated by concomitant ROL therapy (Table 1). This additional drop in body weight may be a consequence of the reduced water intake in this group of mice.

Histology

Figure 2, a and b, shows normal colonic structure and that ROL treatment (twice daily) did not alter colonic architecture, respectively. Colitis evoked by DSS was characterized by severe disruption of tissue architecture, edema, a massive mixed immune cell infiltrate (mononuclear cells, neutrophils, and eosinophils), ulceration and significant areas of complete epithelial denudation, and muscle thickening (Figure 2c). Mice treated with ROL once daily along with DSS experienced the same fate as those in the DSS only group (data not shown). However, animals treated with ROL twice daily showed a significant improvement in colonic histology, ranging from an appearance virtually indistinguishable from control tissue, to colons showing a mild immune cell infiltrate (Figure 2d), and those showing small foci of architectural destruction. Comparison of the tissue damage scores showed that colons from DSS-treated mice had scores in the range of

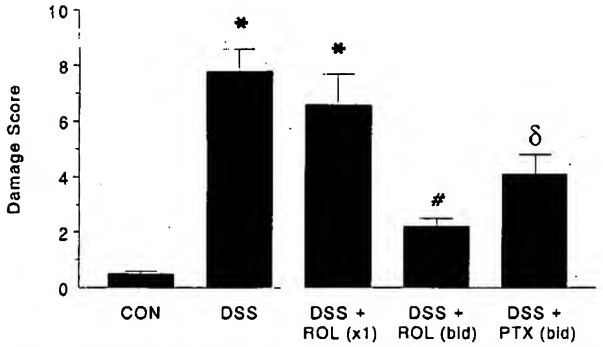

Figure 3. Bar chart showing colonic damage score after exposure to DSS ± PDE inhibitors. Means ± SEM; n = 9 to 15, except DSS + ROL (x1) where n = 4. *,#, δ, statistically different ($P < 0.05$) groups compared to each other and control (CON). See Figure 1 legend for abbreviations and treatment regimen.

5 to 11 (11 is maximum), whereas 11 of 12 animals in the DSS + ROL (twice daily) group had scores of <5; colon from the remaining animal in this group had a damage score of 5 (Figure 3). This reduction in colonic damage is complemented by the reduction in the number of mice displaying evidence of colonic/rectal bleeding (Table 1). Mice treated with DSS + PTX displayed an improvement in colonic histopathology compared to DSS-only mice, but there were still clear signs of edema, inflammatory infiltrate, and muscle thickening (Figure 2e). The damage score of DSS + PTX-treated mice was intermediate between DSS-only and DSS + ROL-treated mice (Figure 3).

MPO Activity

Measurement of MPO activity revealed that DSS colitis was accompanied by an increase in MPO and that this was significantly reduced by concomitant ROL or PTX (twice daily): control = 0.10 ± 0.19; DSS = 1.53 ± 0.3 ($P < 0.05$ compared to control, n = 9 to 20); DSS + ROL = 0.13 ± 0.21; and DSS + PTX = 0.38 ± 0.23 U/mg wet weight of tissue.

Epithelial Function

Baseline Isc, potential difference, and tissue conductance values are shown in Table 2. These gut parameters were not significantly different when tissues from control and DSS colitis mice were compared. In contrast, responses to all three pro-secretory stimuli were significantly reduced in tissues from mice treated with DSS, and this diminished responsiveness was not affected by co-treatment with ROL or PTX (twice daily) (Figure 4).

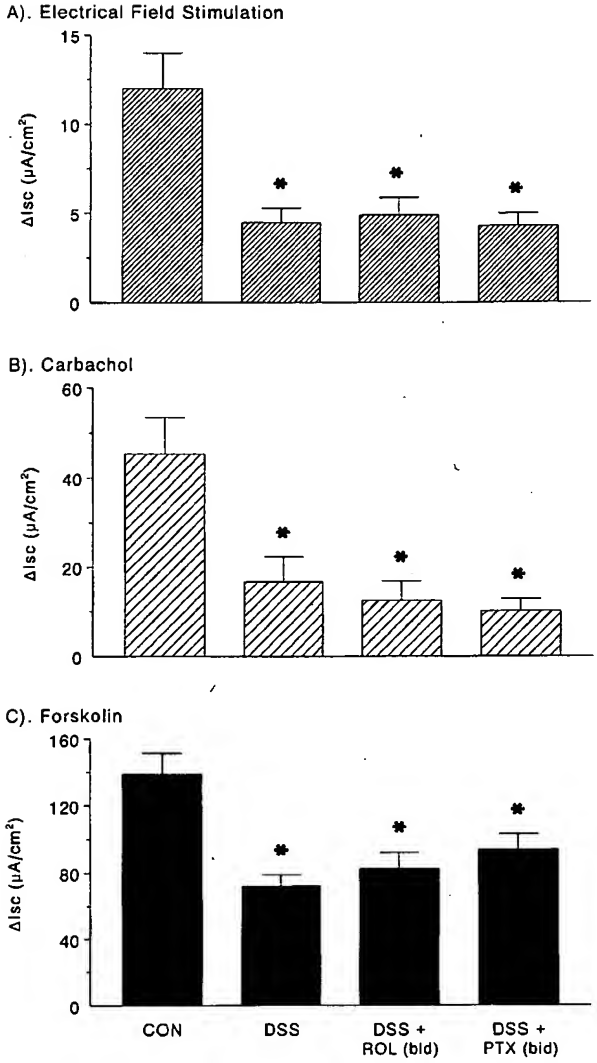

Figure 4. Bar charts showing the change in short-circuit current (ΔIsc) in colonic segments in response to electrical nerve stimulation (**A**), carbachol (10^{-4} mol/L) (**B**), and forskolin (10^{-5} mol/L) (**C**) after 5 days of DSS exposure ± PDE inhibitors. Means ± SEM; n = 9 to 15; *, $P < 0.05$ compared to controls (CON). See Figure 1 legend for treatments.

Cytokine Levels

Enzyme-linked immunosorbent assay of serum samples (n = 6) and tissue homogenates (n = 3) revealed no detectable TNF-α in any of the treatment groups. (The assay protocol used herein has been used to successfully detect serum levels of TNF-α in other studies in our laboratory.)

Table 2. Basal Electrophysiological Parameters in Colonic Segments from Control and DSS-Treated Mice

	Control	4% DSS (5 days)	DSS + ROL	DSS + PTX
Short-circuit current (μA/cm²)	60.3 ± 5.6	46.7 ± 6.7	56.0 ± 10.2	33.9 ± 3.7
Potential difference (mV)	−5.5 ± 0.4	−5.0 ± 0.7	−5.4 ± 0.9	−3.2 ± 0.3
Conductance (mS/cm²)	18.0 ± 0.6	16.2 ± 1.4	20.5 ± 5.2	17.8 ± 1.1
n	15	15	12	9

Mean ± SEM; drugs were administered intraperitoneally twice daily at 5 mg/kg; baseline values were recorded 20 minutes after tissues had been mounted in the Ussing Chambers.

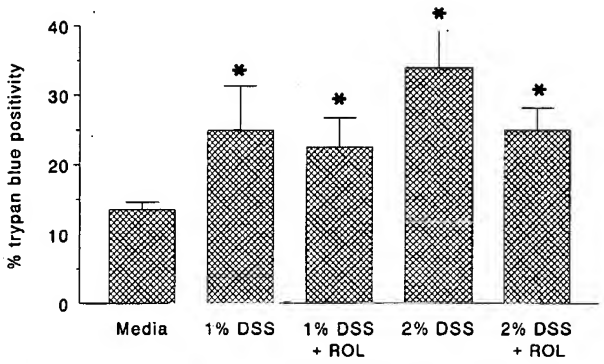

Figure 5. Bar chart showing viability of murine epithelial (IEC.4.1) cells exposed to 1% or 2% DSS for 24 hours *in vitro* ± ROL (10^{-6} mol/L, single addition) as determined by trypan blue dye exclusion, where increased positivity indicates increased cell death. Data are means ± SEM and are presented as percentage of controls cells cultured in media only; $n = 3$ experiments, with three replicates/condition in each experiment. *, $P < 0.05$ compared to controls; positive control of distilled water resulted in 100% positivity (data not shown).

Epithelial Viability

As shown in Figure 5, exposure of a murine epithelial cell line to 1% or 2% DSS for 20 hours caused a two- to threefold increase in cell death that was not altered by concomitant treatment with ROL (10^{-6} mol/L). Similarly, use of the MTT assay as an indicator of epithelial viability (specifically mitochondrial function), revealed a significant reduction in MTT metabolism in epithelial preparations exposed to DSS that was not abrogated by concomitant ROL treatment: control = 0.71 ± 0.04; 1% DSS = 0.58 ± 0.03*, 1% DSS + ROL = 0.58 ± 0.02*, 2% DSS = 0.52 ± 0.0.02*, 2% DSS + ROL = 0.57 ± 0.04* optical density (OD) units ($n = 8$ replicates from a representative experiment, 3 experiments were performed; *$P < 0.05$ compared to control; cells treated with distilled water as a positive control = 0.05 ± 0.01* OD units).

Recovery Studies

The ability of ROL to reduce the histopathology in DSS colitis suggested the possibility that ROL-treated mice might recover more quickly from a 5-day course of DSS. In accordance with previous histological data, ROL + DSS-treated mice examined 3 days after the end of the treatment showed a significant preservation of colonic structure (Figure 2f): damage scores, DSS only = 6.5 ± 0.9 and ROL + DSS = 1.9 ± 0.3 ($n = 3$ and $n = 5$, respectively). Stimulated epithelial ion transport events in colonic segments from mice 3 days after withdrawal of DSS were significantly reduced compared to control tissues (Table 3) and this reduction was similar to, or of a greater magnitude than, that observed from colonic tissue excised at the end of the 5-day DSS treatment (compare Figure 4 and Table 3). However, colon from mice treated with ROL + DSS showed a partial or statistically significant improvement in their responsiveness to prosecretory stimuli (Table 3). In addition, carbachol challenge of colon excised from mice 3 days after the DSS treatment resulted in a drop in Isc (shown as a negative value in Table 3), rather than the expected transient increase in Isc that occurs in tissue from normal mice. In contrast, tissues from mice treated with DSS + ROL all displayed an increase in Isc in response to carbachol, although the magnitude of the response was very variable (6.7 to 65.5 $\mu A/cm^2$).

Discussion

The therapeutic potential of PDE inhibition has been shown in a variety of models of acute and chronic inflammation, and to some extent in trials with patients exhibiting airway inflammation.[1,3,4] Analysis of inhibitors of PDEs have consistently implicated reduced TNF-α synthesis as a key component of their anti-inflammatory activity.[2,5–7] These data suggest that inhibition of PDE activity could be a rational therapy for human idiopathic inflammatory bowel disease, disorders that are often accompanied by elevated TNF-α levels.[9] Indeed, preliminary data from animal studies[18,26] and a trial involving seven patients[27] support this postulate. However, evaluations of the use of PDE inhibitors to treat enteric inflammation have focused on histopathology and primarily neglected assessment of gut function. This study was designed to examine the ability of a PDE4-selective (ie, ROL) and a nonselective PDE inhibitor (ie, PTX) to modulate the structural and functional abnormalities in murine colitis. Thus, using the established DSS model of murine colitis,[10,11] we show that: 1) ROL is more effective than PTX in preventing the DSS-induced colonic histopathology; 2) neither inhibitor affected the ion transport irregularities observed at the end of a 5-day DSS treatment; and 3) ROL-treated mice showed a more rapid recovery in their responsiveness to pro-secretory agents compared to DSS-only treated mice.

Steroids are a mainstay in the treatment of inflammatory bowel disease and although they are very effective, their long-term use results in considerable side effects. Hence, there is a need for the development of other

Table 3. Basal and Stimulated Ion Transport Events in Colon 3 Days after Termination of DSS

	Control	4% DSS (5 days)	DSS + ROL
Baseline Isc ($\mu A/cm^2$)	55.4 ± 14.4	41.0 ± 10.5	83.7 ± 12.6
ΔIsc to electrical nerve stimulation	11.8 ± 3.8	3.7 ± 2.2*	6.3 ± 1.6*
ΔIsc to carbachol (10^{-4} mol/L)	42.1 ± 1.4	−14.3 ± 6.9*	26.7 ± 10.1
ΔIsc to forskolin (10^{-5} mol/L)	152.6 ± 33.3[a]	40.3 ± 23.6[b]	85.9 ± 22.9[a,b]
n	3	3	5

Mean ± SEM; ROL at 5 mg/kg twice a day for the duration of the DSS exposure only.
*, $P < 0.05$ compared to control; superscript letters indicate statistically similar groups using analysis of variance.

therapeutics to combat enteric inflammation, and one such strategy is to enhance immunosuppression by maintaining elevated cAMP levels via inhibition of PDE activity. Twice-daily treatment of mice with low-dose ROL or PTX resulted in significantly less colonic histopathology in animals with free access to 4% (w/v) DSS drinking water. Thus, general inhibition of PDE activity by PTX, or specific targeting of PDE4 by ROL resulted in greater preservation of colonic structure, and a concomitant decrease in tissue MPO levels compared to mice receiving DSS and saline or DSS and drug vehicle. PTX was consistently less effective than ROL in abrogating colonic pathology and because it was used at the same concentration as ROL, a value 20 times less than that used in other PTX studies,[26,28] this suggests that the benefit of either drug is because of inhibition of PDE4, the predominant PDE in immune cells. These data support the use of PDE4 inhibitors as an anti-inflammatory option and confirm recent data showing that inhibition of PDE activity reduces the histopathology associated with colitis.[18,26–28]

Analysis of colonic epithelial ion transport in the DSS colitis model has hitherto not been reported. Many of the mice exposed to DSS displayed a variable diarrhea and the colons of all treated animals were inflamed, often with significant epithelial loss. It is noteworthy that the ROL-treated (twice daily) animals, although showing histological improvement still had macroscopic signs of diarrhea/water imbalance (Table 1) and this treatment did not ameliorate the ion transport (create the driving force for directed water movement) abnormalities caused by exposure to DSS (see below). Baseline Isc across colonic tissue from all DSS-treated mice (regardless of concomitant PDE therapy), was not consistently elevated and ion conductance was within the range for normal tissue, suggesting unaltered permeability. Neither observation fits with the obvious tissue pathology. However, Isc is a composite of all of the active ion transport across the tissue and so additional studies examining specific ion movements in the colitic, and adjacent tissue are required before precise statements regarding the driving forces for water movement can be made. Also, it is inconceivable that epithelial loss would not result in increased permeability and indeed DSS-induced colitis has been shown to increase permeability.[29,30] The absence of a significant increase in conductance observed here is likely because of the use of whole-thickness tissues in the Ussing chamber, such that any increase in epithelial permeability was offset by muscle hypertrophy (compare Figure 2, a and c).

Increases in Isc evoked by all three pro-secretory stimuli were significantly reduced in colonic segments excised from DSS-treated mice. Similar diminished Isc responsiveness has been observed in other animal models of colitis and in tissue resections from patients with inflammatory bowel disease.[31–34] However, neither ROL nor PTX treatment led to any amelioration of the reduced Isc responses, clearly indicating uncoupling of structure and function in this model of colitis. Juxtaposition of these data with the histological portion of the study suggests that either regulation of Isc is more readily altered than derangement of colon structure, or that ion transport and colon structure are regulated by different mechanisms. Similarly, we and others have shown that colonic ion transport can be perturbed in the absence of any evidence of histological abnormalities as defined by light microscopy.[22,32] For instance, colon excised from rats treated with the pro-colitic haptenizing agent TNBS (2,4,6-trinitrobenzenesulfonic acid) seems structurally normal 12 weeks after treatment, and although baseline ion transport and tissue conductance were within the normal range, responsiveness to nerve stimulation and carbachol remained depressed compared to tissues excised from controls.[32] Thus, in assessing the value of any putative anti-inflammatory agent it would seem imperative that structural analysis be complemented by functional studies.

Given the ability of ROL to almost completely prevent DSS-induced histopathology, we postulated that ROL therapy might hasten the recovery process after DSS exposure. In support of this, we observed that whereas colonic tissue from DSS-only treated mice displayed reduced Isc responses to electrical nerve stimulation, carbachol and forskolin 3 days after replacing the DSS water with regular water, the colon from the ROL + DSS-treated animals showed a significant recovery in their secretory responsiveness. These data add further credence to the hypothesis that PDE inhibition may be a valuable adjunct therapy for colonic inflammation.

The mechanism responsible for DSS-induced colitis is unknown and a variety of anti-inflammatory strategies have been used to treat the colitis, at least in terms of the histopathology.[12–16,29,35,36] Furthermore, the induction of colitis in severe combined immunodeficient, $CD4^+$-T cell depleted, and athymic mice by DSS reveals that thymus-dependent T cells are not a prerequisite for the colitis[37,38] This has led to the suggestions that DSS-induced colitis is either a macrophage-driven event[39,40] (which fits with a pivotal role for TNF-α), or because of direct epithelial cytotoxicity/inhibition of proliferation.[37,41,42] Thus, we sought to examine the affect of ROL therapy on TNF-α levels and epithelial viability.

At the end of the 5-day DSS exposure, TNF-α levels were neither detected in the serum nor colonic tissue homogenates from control, DSS, or DSS + ROL-treated mice. Thus, we can provide no evidence in support of inhibition of TNF-α production as a component of the anti-inflammatory activity of ROL in this model system. However, because TNF-α is rapidly mobilized it is possible that ROL did reduce the levels of TNF-α earlier in the treatment regimen. Moreover, although numerous studies have shown that phosphodiesterase (PDE) inhibition reduces macrophage-stimulated TNF-α production,[2,7] it has recently been shown that the reduction in nonsteroidal anti-inflammatory drug-induced enteropathy in the rat by ROL was independent of it's ability to reduce plasma TNF-α levels.[43] Also, the clinical improvement in patients with Crohn's disease treated with PTX did not correlate with reduced tissue TNF-α levels.[27] In addition, PDE inhibition can result in reduced IL-1β, IL-6, IL-8, and IL-12 levels and clearly any, or all of these cytokines may be involved in mediating enteric inflammation.[44–46]

Using the vital stain, trypan blue, and the MTT assay to assess epithelial viability, we found that DSS was directly toxic to the murine IEC.4.1 cell line *in vitro*. These findings are in accordance with other publications.[37] However, simultaneous ROL treatment did not abrogate the DSS cytotoxic effects and we speculate that the beneficial effect of ROL *in vivo* is not because of inhibition of epithelial cell death by directly affecting the epithelial cell.

In summary, we have shown that use of the PDE inhibitors ROL and PTX significantly reduced the histopathology in a murine model of colitis. Neither agent affected the reduced secretory responsiveness that was apparent in colonic tissues examined at the end of the 5-day DSS-treatment period, although ROL therapy did enhance the recovery phase after withdrawal of the colitic stimulus. These findings add to the list of models of colitis that are characterized by perturbations in ion transport and complement a smaller number of investigations illustrating that normal histological appearance is not necessarily reflective of functional normality. We suggest that PDE inhibitors, particularly those targeted at the PDE4 isoform,[47] warrant further assessment as tools to combat enteric inflammation either alone or as part of a combination therapy regimen.[28]

Acknowledgment

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References

1. Torphy TJ: Phosphodiesterase isozymes. Molecular targets for novel anti-asthma agents. Am J Respir Crit Care Med 1998, 157:351–370
2. de Moraes VLG, Singer M, Vargaftig BB, Chignard M: Effects of rolipram on cyclic AMP levels in alveolar macrophages and lipopolysaccharide-induced inflammation in mouse lung. Br J Pharmacol 1998, 123:631–636
3. Doherty AM: Phosphodiesterase 4 inhibitors as novel anti-inflammatory agents. Curr Opin Chem Biol 1999, 3:466–473
4. Teixeira MM, Gristwood RW, Cooper N, Hellewell PG: Phosphodiesterase (PDE) 4 inhibitors: anti-inflammatory drugs of the future? Trends Pharmacol Sci 1997, 18:164–171
5. Klemm P, Harris HJ, Perretti M: Effect of rolipram in a murine model of acute inflammation: comparison with the corticoid dexamethasone. Eur J Pharmacol 1995, 281:69–74
6. Sekut L, Yarnall D, Stimpson SA, Noel LS, Bateman-Fite R, Clark RL, Brackeen MF, Menius JA, Connolly KM: Anti-inflammatory activity of phosphodiesterase (PDE)-IV inhibitors in acute and chronic models of inflammation. Clin Exp Immunol 1995, 100:126–132
7. Miotla JM, Teixeira MM, Hellewell PG: Suppression of acute lung injury in mice by an inhibitor of phosphodiesterase type 4. Am J Respir Cell Mol Biol 1998, 18:411–420
8. Ross SE, Williams RO, Mason LJ, Mauri C, Marinova-Mutafchieva L, Malfait A-M, Maini RN, Feldmann M: Suppression on TNF-α expression, inhibition of Th1 activity, and amelioration of collagen-induced arthritis by rolipram. J Immunol 1997, 159:6253–6259
9. Targan SR, Hanauer SB, van Deventer SJH, Mayer L, Present DH, Braakman T, DeWoody KC, Schaible TF, Rutgeerts PJ: A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor α for Crohn's disease. N Engl J Med 1997, 337:1029–1035
10. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R: A novel method in induction or reliable experimental acute and chronic colitis in mice. Gastroenterology 1990, 98:694–702
11. Cooper HS, Murthy SNS, Shah RS, Sedergran DJ: Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest 1993, 69:238–249
12. Bennett CF, Kronbrust D, Henry S, Stecker K, Howard R, Cooper S, Dutson S, Hall W, Jacoby HI: An ICAM-1 antisense oligonucleotide prevents and reverses dextran sulfate sodium-induced colitis in mice. J Pharmacol Exp Ther 1997, 280:988–1000
13. Tomoyose M, Mitsuyama K, Ishida H, Toyonaga A, Tanikawa K: Role of interleukin-10 in murine model of dextran sulfate sodium-induced colitis. Scand J Gastroenterol 1998, 33:435–440
14. Murthy S, Murthy NS, Coppola D, Wood DL: The efficacy of BATy1015 in dextran sulfate model of mouse colitis. Inflamm Res 1997, 46:224–233
15. Domek MJ, Iwata F, Blackman EI, Kao J, Baker M, Vidrich A, Leung FW: Anti-neutrophil serum attenuates dextran sulfate-sodium-induced colonic damage in the rat. Scand J Gastroenterol 1995, 30: 1089–1094
16. Kojouharoff G, Hans W, Obewrmeier F, Mannel DN, Andus T, Scholmerich J: Neutralization of tumour necrosis factor (TNF) but not IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. Clin Exp Immunol 1997, 107:353–358
17. Mahler M, Bristol IJ, Leiter EH, Workman AE, Birkenmeier EH, Elson CO, Sundberg JP: Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. Am J Physiol 1998, 274: G544–G551
18. Hartmann G, Bidlingmaier C, Siegmund B, Albrich S, Schulze J, Tschoep K, Eigler A, Lehr H-A, Endres S: Specific type IV phosphodiesterase inhibitor rolipram mitigates experimental colitis in mice. J Pharmacol Exp Ther 2000, 292:22–30
19. Nelson JL, Alexander JW, Mao J-X, Vohs T, Ogle CK: Effect of pentoxifylline on survival and intestinal cytokine messenger RNA transcription in a rat model of ongoing peritoneal sepsis. Crit Care Med 1999, 27:113–119
20. Appleyard CB, Wallace JL: Reactivation of hapten-induced colitis and its prevention by anti-inflammatory drugs. Am J Physiol 1995, 269:G119–G125
21. Wallace JL, MacNaughton WK, Morris GP, Beck PL: Inhibition of leukotriene synthesis markedly accelerates healing in a rat model of inflammatory bowel disease. Gastroenterology 1989, 96:29–36
22. Donnelly GAE, Lu J, Takeda T, McKay DM: Colonic epithelial physiology is altered in response to the bacterial superantigen *Yersinia pseudotuberculosis* mitogen (YPM). J Infect Dis 1999, 180:1590–1596
23. McKay DM, Benjamin M, Lu J: $CD4^+$ T cells mediate superantigen-induced abnormalities in murine jejunal ion transport. Am J Physiol 1998, 275:G29–G38
24. McKay DM, Singh PK: Superantigen-activation of immune cells evokes epithelial (T84) transport and barrier abnormalities via interferon-γ and tumour necrosis factor-α. Inhibition of increased permeability, but not diminished secretory responses by transforming growth factor β_2. J Immunol 1997, 159:2382–2390
25. Mosmann TR: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983, 65:55–63
26. Peterson TC, Davey K: Effect of acute pentoxifylline treatment in an experimental model of colitis. Aliment Pharmacol Ther 1997, 11:575–580
27. Katz J, Willis J, Cooper G, Geraci K, Fiocchi C: Treatment of Crohn's disease with pentoxifylline: lack of correlation between clinical improvement and mucosal cytokine levels. Am J Gastroenterol 1999, 94:2644
28. Murthy S, Cooper HS, Yoshitake H, Meyer C, Meyer CJ, Murthy NS: Combination therapy of pentoxifylline and TNFα monoclonal antibody in dextran sulfate-induced mouse colitis. Aliment Pharmacol Ther 1999, 13:251–260
29. Bjork S, Jennische E, Dahlstrom A, Ahlman H: Influence of topical rectal application of drugs on dextran sulfate-induced colitis in rats. Dig Dis Sci 1997, 42:824–832
30. Kitajima S, Takuma S, Morimoto M: Changes in colonic mucosal permeability in mouse colitis induced by dextran sulfate sodium. Exp Anim 1999, 48:137–143
31. Kachur JF, Keshavarzian A, Sundaresan R, Doria M, Walsh R, de las Alas MM, Gaginella TS: Colitis reduces short-circuit current response to inflammatory mediators in rat colonic mucosa. Inflammation 1995, 19:245–259
32. Asfaha S, Bell CJ, Wallace JL, MacNaughton WK: Prolonged colonic

epithelial hyporesponsiveness after colitis: role of inducible nitric oxide synthase. Am J Physiol 1999, 276:G703–G710
33. Hubel KA, Renquist KS: Ion transport in normal and inflamed human jejunum *in vitro*: changes with electrical field stimulation and theophylline. Dig Dis Sci 1990, 35:815–820
34. Crowe SE, Luthra GK, Perdue MH: Mast cell mediated ion transport in the intestine from patients with and without inflammatory bowel disease. Gut 1997, 41:785–792
35. Murthy SNS, Cooper HS, Shim H, Shah RS, Ibrahim SA, Sedergran DJ: Treatment of dextran sulfate sodium-induced murine colitis by intracolonic cyclosporin. Dig Dis Sci 1993, 38:1722–1734
36. Egger B, Procaccino F, Sarosi I, Tolmas J, Buchler MW, Eysselein VE: Keratinocyte growth factor ameliorates dextran sodium sulfate colitis in mice. Dig Dis Sci 1999, 44:836–844
37. Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO: Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology 1994, 107:1643–1652
38. Axelsson L-G, Lanndstrom E, Goldschmidt TJ, Gronberg A, Bylund-Fellenius A-C: Dextran sulfate sodium (DSS) induced experimental colitis in immunodeficient mice: effects in $CD4^+$-cell depleted, athymic and NK-cell depleted SCID mice. Inflamm Res 1996, 45:181–191
39. Shintani N, Nakajima T, Sugiura M, Murakami K, Nakamura N, Kagitani Y, Mayumi T: Proliferative effect of dextran sulfate sodium (DSS)-pulsed macrophages on T cells from mice with DSS-induced colitis and inhibition of effect by IgG. Scand J Immunol 1997, 46:581–586
40. Hirono I, Kuhara K, Hosaka S, Tomizawa S, Golberg L: Induction of intestinal tumors in rats by dextran sulfate sodium. J Natl Cancer Inst 1981, 66:579–583
41. Ni J, Chen S-F, Hollander D: Effects of dextran sulphate sodium on intestinal epithelial cells and intestinal lymphocytes. Gut 1996, 39: 234–241
42. Tessner T, Cohn SM, Schloemann S, Stenson WF: Prostaglandins prevent decreased epithelial cell proliferation associated with dextran sodium sulfate injury in mice. Gastroenterology 1998, 115:874–882
43. Reuter BK, Wallace JL: Phosphodiesterase inhibitors prevent NSAID enteropathy independently of effects on TNFα release. Am J Physiol 1999, 277:G847–G854
44. Neuner P, Klosner G, Schauer E, Pourmojib M, Macheiner W, Grunwald C, Knobler R, Schwartz A, Luger TA, Schwarz T: Pentoxifylline *in vivo* down regulates the release of IL-1β, IL-6, IL-8 and tumour necrosis factor-α by human peripheral blood mononuclear cells. Immunology 1994, 83:262–267
45. Au B-T, Teixeira MM, Collins PD, Williams TJ: Effect of PDE4 inhibitors on zymosan-induced IL-8 release from human neutrophils: synergism with prostanoids and salbutamol. Br J Pharmacol 1998, 123:1260–1266
46. Hasko G, Szabo C, Nemeth ZH, Salzman AL, Vizi ES: Suppression of IL-12 production by phosphodiesterase inhibition in murine endotoxemia is IL-10 independent. Eur J Immunol 1998, 28:468–472
47. Barnette MS, Christensen SB, Essayan DM, Grous M, Parbhakar U, Rush JA, Kagey-Sobotaka A, Torphy TJ: SB 207499 (Ariflo), a potent and selective second-generation phosphodiesterase 4 inhibitor: *in vitro* anti-inflammatory actions. J Pharmacol Exp Ther 1998, 284:420–426

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Specific Type IV Phosphodiesterase Inhibitor Rolipram Mitigates Experimental Colitis in Mice[1]

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ABSTRACT

The specific type IV phosphodiesterase inhibitor rolipram is a potent suppressor of tumor necrosis factor-α (TNF) synthesis. We examined the efficacy of rolipram for the prevention and treatment of experimental colitis. To induce colitis, BALB/c mice received 5% dextran sulfate sodium in their drinking water continuously for up to 11 days. Colitis was quantified by a clinical activity score assessing weight loss, stool consistency, and rectal bleeding (range from 0 to 4); by colon length; by a semiquantitative histologic score (range from 0 to 6); and by detecting TNF concentration in colonic tissue by enzyme-linked immunosorbent assay. In a first protocol, rolipram (10 mg/kg b.wt./day i.p.) was started on the same day as dextran sulfate sodium. Rolipram reduced the clinical activity of colitis (score 1.1 ± 0.3) compared with mice that did not receive rolipram (2.4 ± 0.4; $P = .041$). Rolipram also partially reversed the reduction of colon length (without rolipram, 12.4 ± 0.3 cm; with rolipram, 15.4 ± 0.7 cm; $P = .004$) and improved the histologic score (1.5 ± 0.6 in rolipram-treated mice versus 4.6 ± 0.5; $P = .020$). Rolipram suppressed colonic tissue TNF concentrations. The beneficial effect of rolipram was confirmed in a second protocol in which dextran sulfate sodium exposure was discontinued on day 7 and rolipram was administered from day 8 through day 15. These three series of experiments on a total of 153 mice documented the efficacy of rolipram in both the prevention and treatment of experimental colitis.

In several diseases, the proinflammatory cytokine tumor necrosis factor-α (TNF) forms a necessary element in the chain of pathophysiologic events leading to inflammation. Successful treatment with anti-TNF-antibodies in patients with Crohn's disease (van Dullemen et al., 1995; Stack et al., 1997; Targan et al., 1997; Present et al., 1999), with rheumatoid arthritis (Elliott et al., 1994), and with Jarisch-Herxheimer reaction (Fekade et al., 1996) illustrate anti-inflammatory strategies based on the specific blockade of TNF (Eigler et al. 1997). Among the agents known to inhibit TNF production rather than block its function, attention has focused on cAMP-elevating phosphodiesterase (PDE) inhibitors. The predominant PDE isoenzyme family in monocytes, a main source of TNF production, is the PDEs of type IV. Compared with the nonspecific PDE inhibitor pentoxifylline (Strieter et al., 1988), the specific type IV PDE inhibitor rolipram is a 500-fold more potent inhibitor of TNF synthesis in human mononuclear cells (Semmler et al., 1993).

Rolipram has initially been developed and studied in clinical trials as an antidepressant (Wachtel 1983). Recently, the potential therapeutic use of rolipram in TNF-dependent disease has been demonstrated in several animal models. Rolipram mitigated experimental autoimmune encephalomyelitis in rats (Sommer et al., 1995) and in nonhuman primates (Genain et al., 1995) and decreased clinical activity of experimental arthritis in rats (Nyman et al., 1997; Ross et al., 1997). In mice, rolipram decreased lipopolysaccharide-induced TNF plasma levels (Fischer et al., 1993) and protected from T-cell-mediated liver failure (Gantner et al., 1997).

In humans, the majority of inflammatory bowel disease occurs in two related, albeit clinically and histologically distinct disorders, ulcerative colitis and Crohn's disease. Both diseases are characterized by chronically relapsing inflammation of the bowel of unknown cause. Crohn's disease is characterized by a granulomatous, transmural inflammation of the bowel wall, predominantly in the distal ileum. In contrast, ulcerative colitis is defined by crypt abscesses and ulcerations limited to mucosa and submucosa, associated

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ABBREVIATIONS: TNF, tumor necrosis factor-α; PDE, phosphodiesterase; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; IL, interleukin.

with a prominent inflammatory infiltrate. The mainstay of therapy for inflammatory bowel disease is aminosalicylates and topical and systemic glucocorticoids. Both provide therapeutic benefit and improve quality of life in many patients, but others suffer from recurrent disease despite systemic glucocorticoid therapy. More specific and more effective therapeutic agents with fewer side effects are needed for the permanent control of inflammatory bowel disease.

Several experimental models of inflammatory bowel disease have been described (Kim and Berstad, 1992; Dieleman et al., 1994; Elson et al., 1995). The dextran sulfate sodium (DSS) model of colitis has been recommended for preclinical testing of new pharmacologic compounds for therapy of chronic inflammatory bowel disease (Cooper et al., 1993; Elson et al., 1995). DSS-induced colitis has a number advantages, including its simplicity, the ability to induce inflammatory lesions, and the reproducibility in respect to both time course and severity among individual mice of a given inbred strain. As in Crohn's disease, macrophage activation and TNF production play a key role in DSS-induced colitis. Elevated levels of TNF have been found in the inflamed colons of DSS-treated mice (Dieleman et al., 1994).

Several studies pointed to a necessary mediator function of TNF, particularly in Crohn's disease (Targan et al., 1997; van Deventer, 1997; Present et al., 1999). TNF elevation was more pronounced in Crohn's disease than in ulcerative colitis both in plasma (Murch et al., 1991) and mucosal samples (Murch et al., 1993; Breese et al., 1994). In the present article, we extend our previous in vitro studies on TNF inhibition by rolipram (Semmler et al., 1993; Siegmund et al., 1997; Eigler et al., 1998) to demonstrate that rolipram attenuates the development of experimental colitis and improves recovery of animals with established colitis.

Materials and Methods

Animals and Induction of Colitis. Female BALB/c mice (~6 weeks of age, mean body weight 20 g) were purchased from Harlan Winkelmann GmbH (Borchen, Germany). Mice were kept under standard laboratory conditions at the animal facility at the Medizinische Klinik, Klinikum Innenstadt. Drinking water and food were provided ad libitum. Mice were sacrificed by cervical dislocation under isoflurane anesthesia (Forene; Abbott GmbH, Wiesbaden, Germany). All experiments were approved by the regional animal study committee and are in agreement with the guidelines for the proper use of animals in biomedical research. Animal handling and scoring of colitis were performed in a consequently blinded experimental design.

DSS (molecular mass 40,000 Da) was obtained from ICN Biomedicals GmbH (Eschwege, Germany) and dissolved in distilled water. Colitis was induced by providing drinking water containing 5% DSS (w/v) for 7 to 11 days as indicated. Control mice received distilled water.

Clinical Activity Score. Colitis was quantified with a clinical score assessing weight loss, stool consistency, and bleeding (measured by guaiac reaction, hemoccult) as described previously (Cooper et al., 1993). No weight loss was counted as 0 points, weight loss of 1 to 5% as 1 point, 5 to 10% as 2 points, 10 to 20% as 3 points, and >20% as 4 points. For stool consistency, 0 points were given for well formed pellets, 2 points for pasty and semiformed stools that did not stick to the anus, and 4 points for liquid stools that did stick to the anus. Bleeding was scored 0 points for no blood in hemoccult, 2 points for positive hemoccult, and 4 points for gross bleeding. These scores were added and divided by 3, forming a total clinical score that ranged from 0.0 (healthy) to 4.0 (maximal activity of colitis).

Colon Length, Histologic Scoring, and Mean Cross-Sectional Area. Postmortem, the entire colon was removed from the cecum to the anus and placed without tension on cellulose. Colon length was measured as an indirect marker of inflammation.

Rings of the ascending, transverse, and descending part of the colon were fixed in 10% formalin and embedded in paraffin for histologic analysis. Sections were stained with hematoxylin/eosin. Histologic scoring was performed by a pathologist (0 to 3 points for infiltration of inflammatory cells plus 0 to 3 points for the degree of tissue damage). For infiltration of inflammatory cells, rare inflammatory cells in the lamina propria were counted as 0; increased numbers of inflammatory cells in the lamina propria as 1; confluence of inflammatory cells, extending into the submucosa as 2; and a score of 3 was given for transmural extension of the infiltrate. For tissue damage, no mucosal damage was counted as 0, discrete lymphoepithelial lesions were counted as 1, surface mucosal erosion was counted as 2, and a score of 3 was given for extensive mucosal damage and extension through deeper structures of the bowel wall. The combined histologic score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage).

For the image analysis of cross-sectional areas, glass slides were imported into Photoshop (Adobe Systems Incorporated, San Jose, CA) on an Apple computer (G3; Apple Computer, Inc., Cupertino, CA) with a kodachrome slide scanner (Nikon LS 1000). Kodachrome frames were opened on one side to allow introduction of the glass slide. Three entire cross sections of each colon part were selected with the "magic wand tool" in the Photoshop toolbox (Lehr et al., 1997). The cross-sectional area of the three bowel sections was quantified with the "histogram tool" (in number of pixels) and was transformed into square millimeters with comparative measurements of a micrometer slide.

Treatment with Rolipram. Rolipram (0.5 mg), kindly supplied by Schering AG (Berlin, Germany), was diluted in 1 ml of distilled water by heating the solution for 30 s at 60°C and then cooling it at room temperature for 2 min. This was repeated until rolipram was totally dissolved. The solution was then frozen into aliquots of 2 ml at −80°C. This proved to be the most reliable protocol to dissolve a relatively high concentration of rolipram for a low total injection volume of 200 μl. Rolipram (5 mg/kg b.wt. b.i.d.) was injected two times per day i.p. with a total injection volume of 200 μl each. Control mice were injected with 200 μl of 0.9% NaCl. To test the therapeutic efficacy of rolipram, two protocols were used: 1) in the concurrent treatment protocol (prevention of colitis), DSS was administered for up to 11 days with rolipram therapy starting the same day as DSS; and 2) in the delayed treatment protocol (treatment of established colitis), colitis was first induced by DSS administration from day 1 to day 7, rolipram therapy was then started at day 8, and was continued up to day 15.

Colon Cytokine Extraction. Strips (~4 cm) of colon from DSS-exposed mice with or without rolipram treatment were weighed, vigorously vortexed for 1 min in 100 μl of 0.01 M PBS (Boehringer Mannheim, Ingelheim, Germany), and centrifuged at 10,000*g* at 4°C for 15 min. TNF was quantified in the eluate with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, MA) according to the manufacturer's instructions. The lower limit of detection of the assay is 50 pg/ml.

Statistical Analysis. All data are expressed as means ± S.E. Statistical significance of differences between treatment and control groups was determined by the unpaired two-tailed Student's *t* test. Where applicable, *P* values were corrected by the Bonferroni method for three independent comparisons (clinical score, colon length, and histologic degree of inflammation). Differences were considered statistically significant for $P < .050$. Statistical analyses were performed with StatView 512 software (Abacus Concepts, Berkeley, CA).

Results

Characteristics of Colitis. Treatment of BALB/c mice with 5% DSS in drinking water for 7 or 11 days resulted in clinical, gross, and histologic signs of colitis that resolved gradually when DSS administration was discontinued (Figs. 1–5). Mice produced loose stool or diarrhea, occult or gross rectal bleeding, and lost weight. After 11 days, the colon length of DSS-treated mice was 12.4 ± 0.3 cm (n = 5) compared with 17.4 ± 0.3 cm (n = 4; $P < .001$) in healthy controls. This has been described as a morphologic parameter of colon inflammation (Okayasu et al., 1990).

Histologic examination of colon sections from control mice showed no signs of inflammation (Figs. 1A and 2A). Histology of colon sections of DSS-treated mice revealed multiple erosive lesions and inflammatory cell infiltration composed mainly of macrophages with fewer lymphocytes and occasional eosinophils and neutrophils (Fig. 1, B and C). In some colon sections, particularly of advanced lesions, regeneration of epithelium were noted in the mucosa. The histologic score (assessing the extent of infiltration of inflammatory cells and the degree of tissue damage, range from 0 to 6) was determined as the mean score of sections of the ascending colon, transverse colon, and descending colon, each section evaluated separately. The most severe lesions were observed in the transverse and the descending colon. After 11 days of continuous DSS administration, a histologic severity score of 4.6 ± 0.5 (n = 14) was reached (Fig. 6).

Mice studied at different time points during DSS treat-

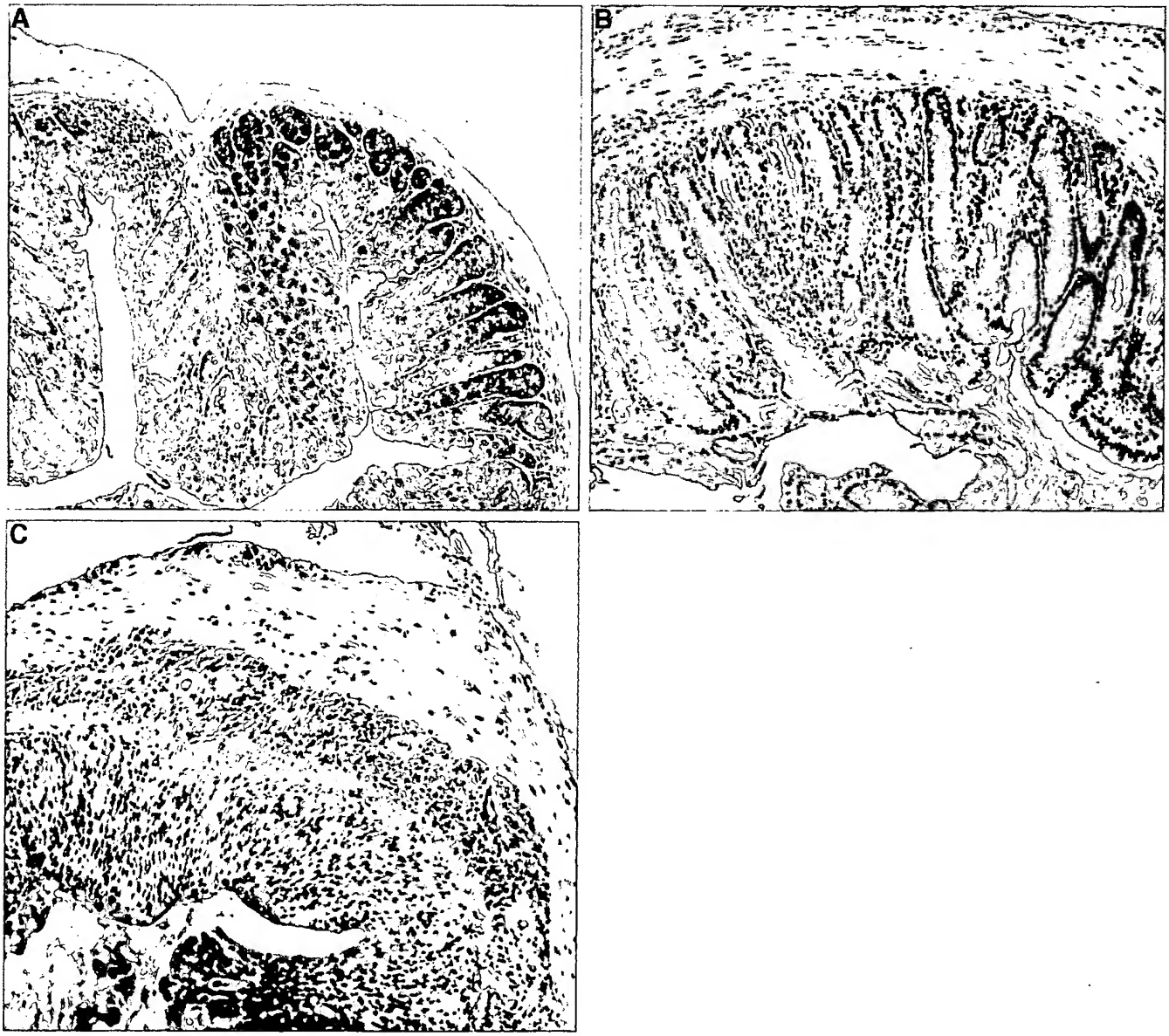

Fig. 1. Histologic characteristics of DSS-induced colitis. A, normal colonic mucosa with regularly formed colonic folds covered by intact mucosa. Only occasional leukocytes are present in the lamina propria of the mucosa. B, a focal accumulation of lymphocytes, histiocytes, and fewer neutrophils compared with (A). Although the surface layer of the mucosa is still intact, there is marked crypt destruction by the inflammatory infiltrate. The inflammatory process is limited to the mucosa and submucosa. C, severe colonic inflammation, characterized by a dense inflammatory cell infiltrate in all colonic wall structures and widespread mucosal sloughing. Note the circumscription of the destructive process, immediately adjacent to normal appearing mucosa. Magnification of the images is 130-fold for (A) and (C), and 325-fold for (B).

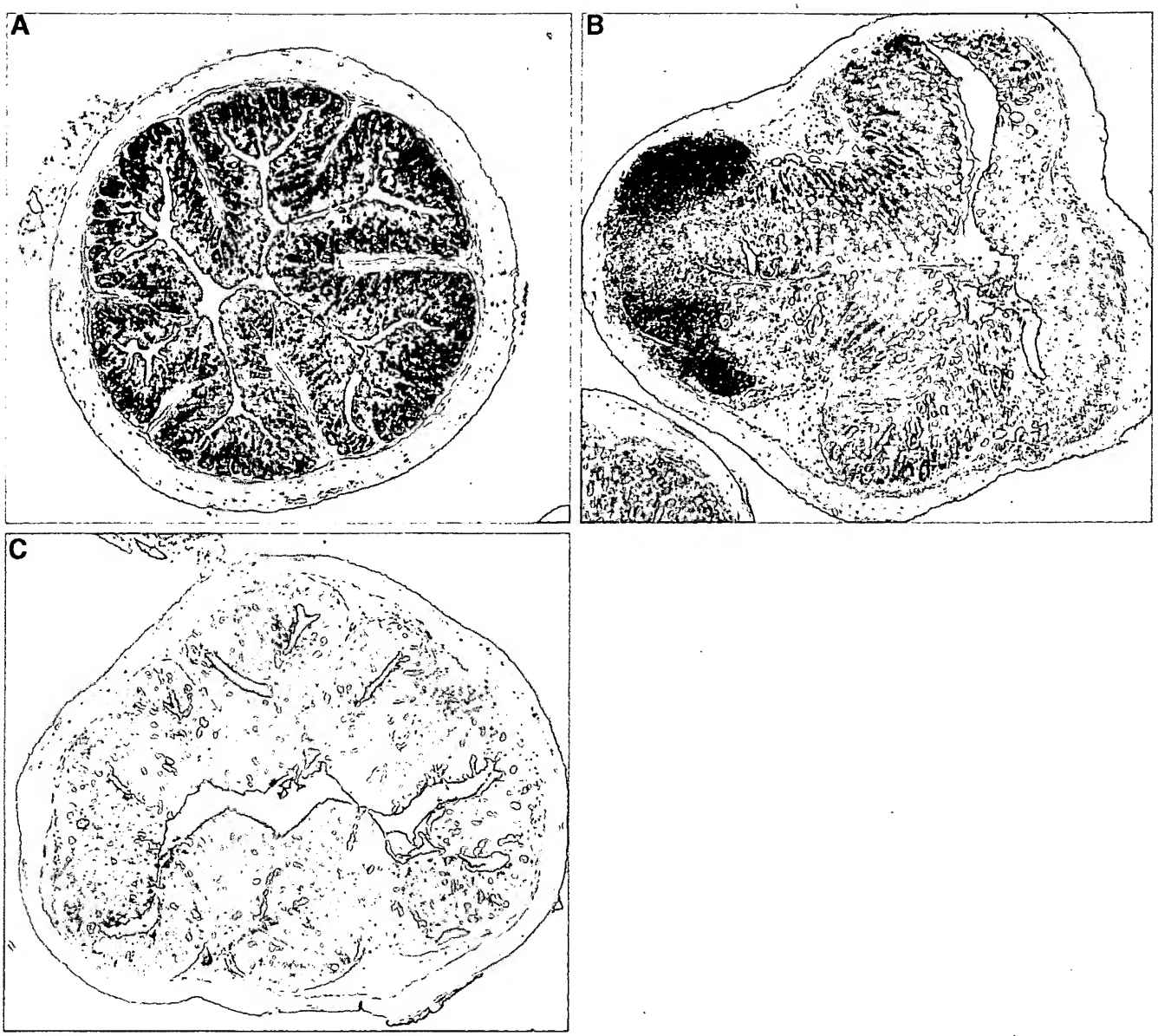

Fig. 2. Influence of rolipram on the histologic manifestation of DSS-induced colitis. Shown are representative cross sections of the transversal colon in control mice without DSS (A) and in DSS-treated mice treated with i.p. injections of 0.9% NaCl (B) or rolipram [10 mg/kg b.wt., (C)] at day 11. Note the regional mucosal destruction in DSS-treated mice (B), which is effectively reversed by rolipram (C). In rolipram-treated mice, there is still some degree of edema, but no cross-mucosal damage. Also note the difference in colon cross-sectional area, which is partially reversed by rolipram. Magnification of the images is 52-fold.

ment (days 0 to 7) and after DSS discontinuation (days 8 to 15) developed increasing histologic signs of colitis over time (Fig. 3). Although mice improved clinically after discontinuation of DSS administration at day 7 (clinical score at day 8 was 2.1 (n = 2) compared with 0.8 at day 15 (n = 7; P = .008; data not shown), on the histologic level no decrease of cellular infiltration and tissue damage could be found until day 15 (Fig. 3). The apparent lack of improvement on the histologic level may be due to cellular infiltration involved in tissue regeneration during the first days of recovery.

Prevention of Colitis with Rolipram. In the concurrent treatment protocol, we tested the effect of rolipram on the prevention of DSS-induced colitis. Mice were administered 5% DSS in their drinking water and were injected i.p. with rolipram or with 0.9% NaCl for a total of 11 days. This protocol was studied in two independent experimental series. The first series comprised 14 mice. Mice fed with DSS developed clinical signs of colitis expressed by an activity score >0.5 starting from day 4 (Fig. 7). Intraperotineal injection of rolipram in a dose of 10 mg/kg b.wt. daily did not retard onset of colitis during the first 6 days of DSS administration. After that, it significantly reduced the progression of colitis as expressed by a lower clinical activity score (1.1 ± 0.3; n = 5 in rolipram-treated mice compared with 2.4 ± 0.4; n = 5 in NaCl controls; P = .041; day 11) (Fig. 7). Each of the three clinical parameters assessed in the clinical score was beneficially influenced by rolipram (body weight, 19.5 g ± 1.5 g in rolipram-treated mice versus 18.0 ± 0.9 g in NaCl-treated controls; stool consistency score, 0.0 ± 0.0 versus 1.2 ± 0.5; rectal bleeding score, 0.8 ± 0.8 versus 2.4 ± 1.0). Control

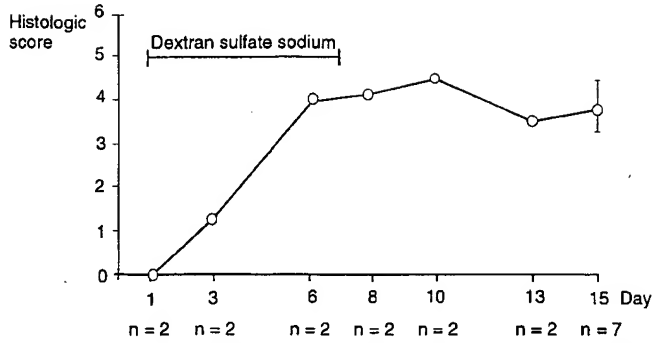

Fig. 3. Time course of DSS-induced colitis at the histologic level. Mice were treated with 5% DSS in drinking water for 8 days. At the indicated time points, mice were sacrificed and sections of the ascending, transverse, and descending colon were examined and scored for cellular infiltration and tissue damage. The magnitude of the histologic score reflects the degree of colitis (0, no changes, to 6, severe tissue damage and cell infiltration). Data represent the mean ± S.E. histologic score (average of the scores of a ascending, transverse, and descending section) of two mice (days 1 to 13) or of seven mice (day 15). Mice without DSS treatment had no signs of colitis (histologic score 0).

mice (without DSS) treated with 0.9% NaCl or rolipram i.p. developed no signs of colitis (Fig. 7). At day 11, all mice were sacrificed. Of the DSS-treated mice, those with rolipram therapy had a longer colon (15.4 ± 0.7 cm) than NaCl controls (12.4 ± 0.3 cm; $P = .004$). Thus, rolipram had partially reversed the colon shortening induced by DSS compared with mice with normal drinking water (17.4 ± 0.3 cm). This argued for a lower extent of inflammation, which was confirmed by histologic examination in the rolipram group (Fig. 2C) compared with the NaCl group (Fig. 2B). In DSS-treated mice, rolipram decreased the histologic score compared with NaCl-treated control mice (1.5 ± 0.6 versus 4.6 ± 0.5; $P = .020$; Fig. 6).

The second experimental series studied with concurrent rolipram treatment comprised 36 animals. The beneficial effect of rolipram was confirmed by clinical [1.8 ± 0.6 in rolipram-treated mice ($n = 14$) versus 3.7 ± 0.3 in control mice ($n = 14$; $P = .021$; day 11; data not shown] and histologic scoring (1.9 ± 0.6 versus 4.3 ± 0.4; $P = .014$). The colon lengths in this series were 12.8 ± 0.4 versus 10.0 ± 0.3 cm; $P < .001$). In this experimental series, a further indicator of

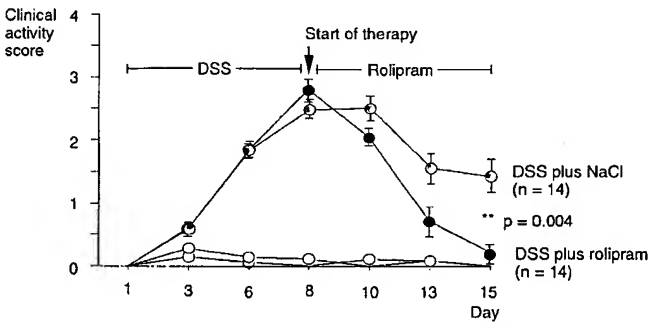

Fig. 4. Accelerated remission of established colitis by rolipram. Mice were treated with DSS in drinking water for 7 days. Rolipram treatment (10 mg/kg b.wt. daily) was started at day 8 after discontinuation of DSS. The degree of colitis was quantified by the clinical score (assessing weight loss, stool consistency, and bleeding; see *Materials and Methods*). Mice with rolipram treatment (●) recovered earlier than mice without rolipram treatment (gray circles; $P = .022$ at day 15; unpaired t test). Control mice without DSS exposure injected with either rolipram or 0.9% NaCl developed no signs of colitis. Scores are depicted as means ± S.E. DSS groups, $n = 14$; control groups, $n = 6$).

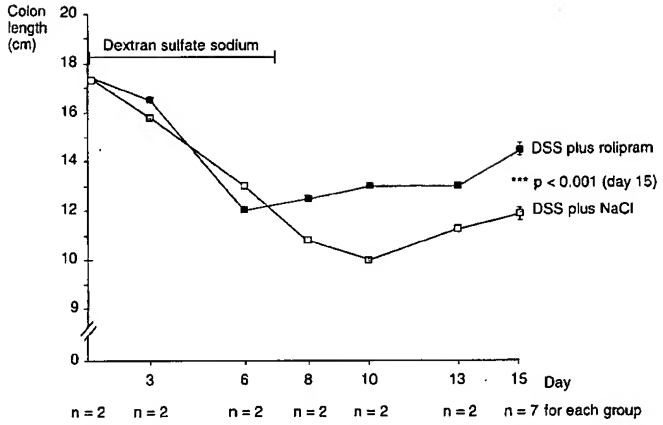

Fig. 5. Reduction of colon length in DSS-induced colitis with and without rolipram treatment. Mice were treated with DSS in drinking water for 7 days. Rolipram treatment (10 mg/kg b.wt. daily) was started at day 8 after discontinuation of DSS. At the indicated time points, two mice of both DSS groups and one mouse of both control groups (with or without rolipram, respectively) were sacrificed and the length of the colon was measured. At day 15, the remaining 7 mice of both DSS groups and the remaining 3 mice of both control groups were examined. DSS-induced colitis lead to shortening of the colon as a marker of inflammation. Rolipram treatment partially reversed this shortening ($P < .001$ at day 15; unpaired t test). Values are depicted as the mean length of the colon in each group. Error bars indicate S.E.M.

inflammation, the mean cross-sectional area of the colon wall was assessed with computer-based image analysis in nine colonic sections from each colon (Lehr et al., 1997). The mean area was higher in DSS-exposed mice (2.1 ± 0.2 mm^2) compared with mice with normal drinking water (1.3 ± 0.2 mm^2; $P = .011$). In the rolipram-treated DSS-exposed mice, this increased colon wall thickness was completely reversed (1.2 ± 0.1 mm^2; $P < .001$; Fig. 2).

For measurement of colonic TNF concentration, the colon from 15 mice of a third experimental series treated as described above was obtained at day 11 (Fig. 8). Eluate from colonic tissue of DSS-exposed mice without rolipram treatment had the highest TNF concentration of 201 ± 39 pg/mg (wet weight) colonic tissue ($n = 4$) compared with 115 ± 25 pg/mg colonic tissue ($n = 7$) in rolipram-treated mice. In the noninflamed colon of control mice receiving rolipram alone, TNF was 97 pg/mg colonic tissue ($n = 2$) and in mice receiving 0.9% NaCl TNF was 97 pg/mg colonic tissue ($n = 2$).

Treatment of Established Colitis with Rolipram. To further evaluate the therapeutic value of rolipram, we studied the effect of rolipram on preexisting colitis (delayed treatment protocol). Colitis was induced by the administration of DSS for 7 days. On day 8, after discontinuing DSS administration, rolipram therapy was started. A total of 88 mice was included in two independent series of studies. The first series was designed to assess clinical score and postmortem morphologic parameters at defined time points during resolution of colitis. In the second series, clinical parameters were followed until complete resolution of clinical colitis in treatment and control groups.

The first study included 54 mice, with 7 or 3 mice in each of the four treatment groups available for morphologic examination at the end of the study. The other mice were sacrificed at the indicated time points during the study.

On the first day of treatment (day 8), the groups designated to receive rolipram or 0.9% NaCl, respectively, had

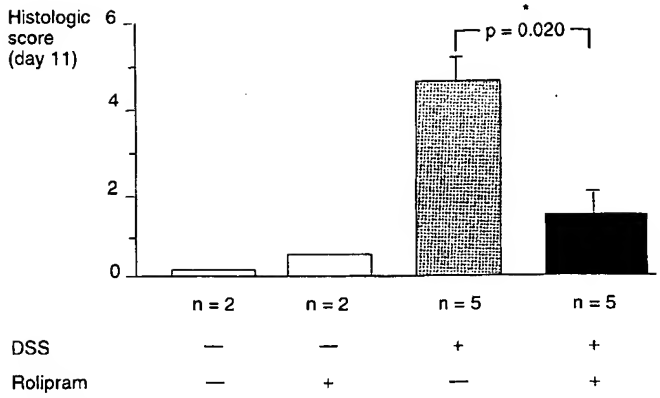

Fig. 6. Reduction of histologic signs of colon inflammation by rolipram. Mice were treated with 5% DSS in drinking water for 11 days and were given rolipram (10 mg/kg b.wt./day i.p.; $n = 5$) or 0.9% NaCl ($n = 5$). At day 11, mice were sacrificed and colon sections were stained and the histologic score (degree of inflammation: 0, no changes, to 6, extensive cell infiltration and tissue damage) was determined in a blinded fashion by a pathologist. In the rolipram-treated group, the histologic score was lower compared with mice given 0.9% NaCl ($P = .020$; unpaired t test). Control mice without administrating DSS developed no colitis. Scores are depicted as means ± S.E.

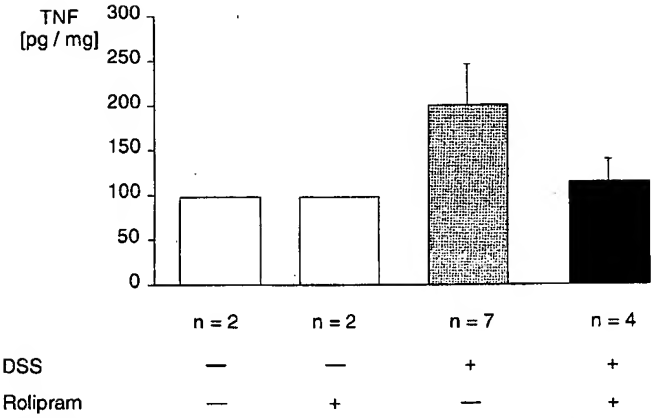

Fig. 8. Suppression of colonic TNF concentration by rolipram. Mice were treated with 5% DSS in drinking water for 11 days. At day 11, the colon was removed, weighed, vortexed for 1 min in 100 μl of PBS, and centrifuged at 10,000*g* for 15 min as described in *Materials and Methods*. TNF was quantified in the eluate by ELISA. Maximal TNF concentration could be measured in colonic tissue of DSS-treated mice (201 ± 39 pg/mg colonic tissue; $n = 4$) compared with rolipram-treated mice (115 ± 25 pg/mg colonic tissue; $n = 7$). In control mice colon, we observed the lowest concentrations, respectively (97 pg/mg colonic tissue; $n = 2$ in each control group).

developed similar clinical activity of colitis (2.8 ± 0.2 in the rolipram group; 2.5 ± 0.2 in the NaCl group; N.S.; Fig. 4). After discontinuation of DSS administration, the clinical score in the control group declined gradually until day 15 (1.4 ± 0.3; $n = 7$). The rolipram-treated mice recovered faster as demonstrated by an earlier decrease and a lower clinical score at day 15 (0.3 ± 0.1; $n = 7$; $P = 0.003$).

Changes of the colon length reflected the clinical course (Fig. 5). At the indicated time points, two mice in each group were sacrificed. Beginning at day 10, the colon length in the rolipram group showed an earlier increase (indication of decreased inflammation) compared with the control group. From day 0 to 13, the variation is due to the low number of mice at each time point (two mice in each group). At day 15, all remaining mice were sacrificed (seven mice each in groups with DSS, three mice each in both groups without DSS). The colon length in the rolipram group was significantly longer

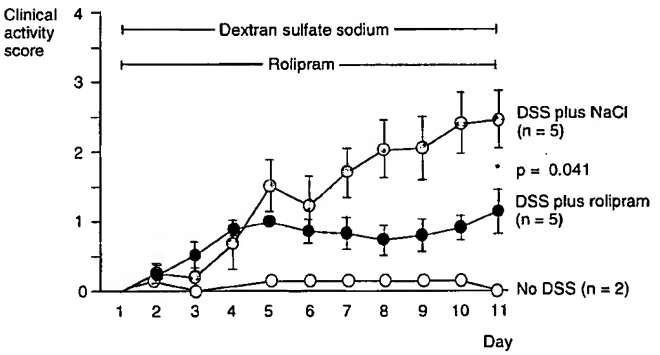

Fig. 7. Mitigation of DSS-induced colitis by rolipram. Mice were treated with 5% DSS in drinking water for 11 days. The degree of colitis was quantified by the clinical score assessing weight loss, stool consistency, and bleeding (range from 0, healthy, to 4, maximal activity; see *Materials and Methods*). Rolipram treatment (10 mg/kg b. wt. b.i.d. i.p.; ●; $n = 5$) beginning the same day as DSS administration reduced the development of colitis compared with mice 0.9% NaCl treatment (gray circles; $n = 5$; one mouse died on day 8; $P = .041$; unpaired t test). Control mice ($n = 2$) without DSS treatment but injection of rolipram (○; $n = 2$) or of 0.9% NaCl (data not shown; $n = 2$) showed no clinical signs of colitis. Scores are depicted as means ± S.E.

than in DSS-fed mice given 0.9% NaCl (14.4 ± 0.4 versus 11.9 ± 0.3 cm; $P < .001$). In the control groups without DSS exposure, the colon length in mice treated with rolipram (17.8 ± 0.2 cm) was unchanged compared with mice injected with 0.9% NaCl (17.5 ± 0.5 cm; data not shown).

On the histologic level, no differences between rolipram-treated mice and the control group were found (histologic score 4.1 ± 1.1 in the rolipram group versus 4.3 ± 0.7 in the control group). For the mean cross-sectional area of the colon, there was only a trend to lower values in the rolipram group (2.8 ± 0.3 mm^2) compared with the control group (3.4 ± 0.3 mm^2; $P = .195$). Both were markedly higher than in mice without DSS treatment (1.8 ± 0.1 mm^2; $P = .010$), reflecting inflammation in the DSS-treated mice.

In the second series with the delayed treatment protocol, clinical parameters were followed until complete resolution of clinical signs of colitis. This study included 34 mice, 14 in both DSS-exposed groups and 3 mice in both groups without DSS. After 7 days, the groups designated to receive rolipram or 0.9% NaCl, respectively, had developed the same extent of colitis as in the first series (clinical activity score of the rolipram group, 2.1 ± 0.3, and for the NaCl group, 2.3 ± 0.3; data not shown). Confirming the results of the first series, rolipram-treated mice recovered earlier than control mice. The difference in the clinical activity score between both groups was maximal at day 11 (rolipram group, 1.3 ± 0.3; NaCl group, 2.4 ± 2.1; $P = .005$) and declined to nonsignificance until day 15 (rolipram group, 0.5 ± 0.2; NaCl group, 0.9 ± 0.3; $P = .301$). At day 15, the colon length of the rolipram-treated mice was significantly longer (15.2 ± 0.4 cm) than the colon length of untreated mice (13.0 ± 0.3 cm, $P < .001$; controls without DSS, 17.5 ± 0.3 cm). Differences in the individual scores of body weight, rectal bleeding, and stool consistency between both groups are summarized in Fig. 9B. Rolipram significantly decreased rectal bleeding and the appearance of loose stool. There was a nonsignificant trend to higher body weight in the rolipram group ($P = .18$).

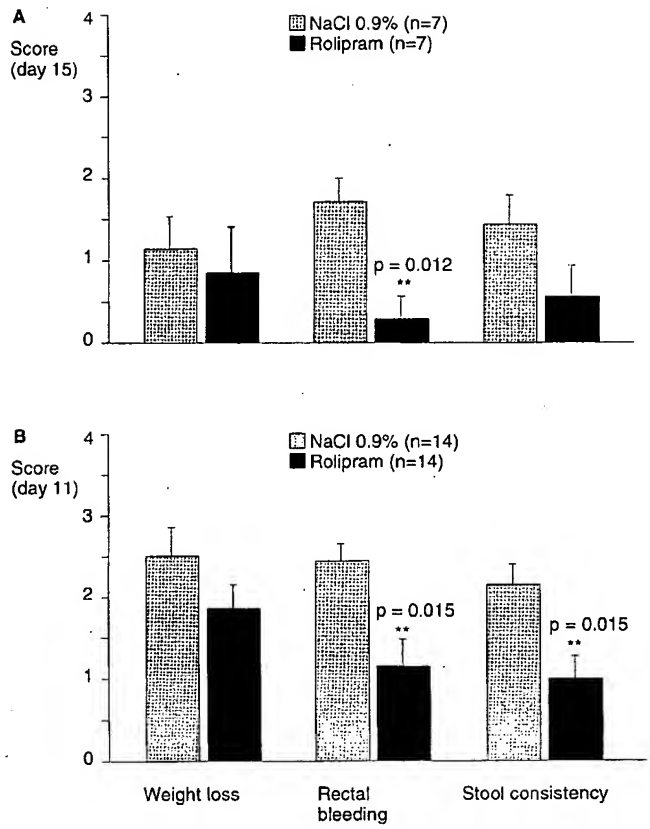

Fig. 9. Beneficial effect of rolipram on weight loss, stool consistency, and bleeding. Clinical parameters in two independent series of experiments with 54 mice (A) and 34 mice (B) examining the effect of rolipram in the model of established colitis. Mice were treated with 5% DSS for 7 days before starting therapy with rolipram (10 mg/kg b.wt./day) on day 8. Clinical signs of colitis are summarized for the final day of the first series [day 15 (A), time course of composite clinical score of this study shown in Fig. 8] and for the day of maximal difference between treatment groups in the second series [day 11 (B); in this series, no significant differences at day 15]. Loss of body weight, stool consistency, and bleeding were semiquantified by scores (see *Materials and Methods*). In the first series, one mouse in the DSS group without rolipram died at day 8 for unknown reasons. Data are shown as means ± S.E. Statistical evaluation was performed using the unpaired t test and P values were Bonferroni corrected.

The results are in accordance with the findings of the first series regarding individual clinical parameters (Fig. 9A).

Discussion

Summary of Results. Type IV PDE inhibitors have been identified as a potential therapeutic principle for asthma (Turner et al., 1994) and multiple sclerosis (Genain et al., 1995; Sommer et al., 1995) because they are potent inhibitors of diverse leukocyte functions. In this study, we present evidence that the specific type IV PDE inhibitor rolipram abrogates experimental colitis in mice. A total of 138 female BALB/c mice was included in our studies. Administration of DSS in drinking water induced clinical and histologic signs of colitis. In agreement with published studies, we found reproducible and interindividually similar degrees of colitis in DSS-treated mice.

Rolipram therapy was effective for both prevention of colitis (Figs. 6 and 7) and therapy of established colitis (Figs. 4 and 5). In the prevention model, rolipram administered in parallel with DSS did not inhibit or delay onset (~day 4) but decreased aggravation of colitis at later time points (between days 5 and 11; Fig. 7). In accordance with the clinical and histologic results, rolipram suppressed TNF synthesis in the colon of DSS-fed mice. The model of delayed treatment was designed to test the efficacy of rolipram as therapy of preexisting colitis. Rolipram improved recovery of mice from established colitis after discontinuation of DSS treatment. Signs of colitis decreased earlier in rolipram-treated mice than in mice without therapy (Fig. 4).

DSS Model. Several experimental models of inflammatory bowel disease have been described (for review, see Elson et al., 1995). The DSS model of colitis has been recommended for preclinical testing of new pharmacologic compounds for therapy of chronic inflammatory bowel disease (Cooper et al., 1993; Elson et al., 1995). DSS-induced colitis has a number of advantages, including its simplicity, the ability to induce both acute and chronic inflammatory lesions, the high degree of uniformity of the lesions, and the reproducibility in respect to both time course and severity among individual mice of a given inbred strain. This uniformity and reproducibility is not achieved in several other experimental models of inflammatory bowel disease (Elson et al., 1995).

Side Effects of Rolipram. In general, therapy with rolipram at a dose of 10 mg/kg b.wt./day was well tolerated by the mice. Immediately after injection of rolipram, mice showed reduced motility, but they returned to normal motoric behavior after a few minutes. These changes could not be attributed to the i.p. injection of fluid because changes in the behavior did not occur in control mice injected with 0.9% NaCl. In agreement with previous in vivo studies (Turner et al., 1994; Genain et al., 1995; Gantner et al., 1997; Nyman et al., 1997; Ross et al., 1997) with the same (10 mg/kg b.wt./day) or lower doses of rolipram, we observed no major side effects. For higher doses, side effects in nonhuman primates include vomiting, salivation, and mouth scratching, none of which was observed in the present study (Genain et al., 1995).

Fasting has recently been described to have some protective effect during development of DSS-induced colitis (Savendahl et al., 1997). In our study, in control mice without DSS, injection of rolipram did not influence body weight. In contrast, rolipram-treated DSS mice had higher body weight compared with mice without rolipram. Therefore, it is unlikely that rolipram exerts anti-inflammatory activity by decreasing food intake as a possible mechanism of action.

Cellular Effects of Rolipram. The anti-inflammatory activity of rolipram depends on its direct action on leukocytes. We and others have shown that rolipram strongly inhibits TNF production in monocytes and macrophages (Schade and Schudt, 1993; Semmler et al., 1993; Prabhakar et al., 1994; Seldon et al., 1995; Barnette et al., 1996). We have demonstrated that the synthesis of the anti-inflammatory cytokine interleukin (IL)-10 is enhanced by rolipram (Eigler et al., 1998) and that exogenous IL-10 acts synergistically with rolipram in decreasing TNF production (Siegmund et al., 1997). Furthermore, rolipram inhibits IL-2-mediated proliferation of primary T cells but not IL-2 production itself (Essayan et al. 1994) and inhibits γ-interferon synthesis of T cells (Essayan et al., 1994; Sommer et al., 1995). These anti-inflammatory characteristics of rolipram act in concert to effectively inhibit the inflammatory response in

vivo (Turner et al., 1994; Genain et al., 1995; Sommer et al., 1995; Gantner et al., 1997).

Determination of Endpoints in Colitis Model. We quantified clinical activity with a scoring system that has been described to be a reliable marker of pathologic changes (Cooper et al., 1993). The clinical score was determined in a blinded fashion to exclude bias by the examining person. Shortening of the colon as a morphologic parameter for the degree of inflammation correlates well with pathologic changes (Okayasu et al., 1990). In our studies, the length of the colon proved to be an easily determined and consistent marker of colitis. Histologic examination was performed blinded and included the degree of infiltration by inflammatory cells in the mucosa and the degree of tissue damage. In our study, a histologic score calculated from these two markers was found to parallel clinical changes during induction of colitis.

TNF-α and Colitis. There is evidence that TNF plays a central role in inflammatory bowel disease (for review, see van Deventer, 1997; Sandborn and Hanauer, 1999). The therapeutic benefit of TNF inhibition in Crohn's disease has been shown in clinical studies with chimeric anti-TNF antibodies (van Dullemen et al., 1995; Stack et al., 1997; Targan et al., 1997; Present et al., 1999). Although proving the principle of targeting TNF in inflammatory bowel disease, efficacy of anti-TNF antibody therapy may decrease with time because of the formation of anti-idiotype antibodies. Furthermore, the development of antinuclear antibodies has been observed with prolonged anti-TNF antibody therapy in patients with rheumatoid arthritis (Elliott et al., 1994).

Anti-TNF antibodies also have been tested in DSS-induced colitis. Neutralization of TNF but not of IL-1 reduced inflammation in the chronic model of DSS-induced colitis in mice (Kojouharoff et al., 1997). In another study, treatment with anti-TNF antibody failed to prevent onset of colitis in the acute model of DSS-induced colitis (Olson et al., 1995). These primarily contradictory results may be due to the protective effect of TNF against bacterial invasion in intact mucosa.

PDE Inhibition and TNF-α. Intracellular concentrations of cAMP increase either as a consequence of receptor-triggered adenylyl cyclase activation or by decreased activity of PDEs. Cyclic nucleotide PDEs have been classified into nine distinct families with several subgroups (Beavo, 1995). Because the predominant PDE family in monocytes, the main source of TNF, is PDE IV (Seldon et al., 1995; Souness et al., 1997), specific type IV PDE inhibitors such as rolipram have proven high potency in suppressing TNF synthesis. Activators of adenylyl cyclase, such as prostaglandin E_2 and prostacyclin analogs, synergize with PDE inhibitors both in increasing cAMP levels and in suppressing TNF synthesis (Sinha et al., 1995). One may speculate that PDE inhibitors given systemically may exert their maximal TNF-suppressing effect in tissue containing prostaglandin E_2 such as the inflamed mucosa of inflammatory bowel disease.

Rolipram In Vivo. In humans, the specific type IV PDE inhibitor rolipram has been extensively studied as an antidepressant. However, it has not been marketed because of the lack of an additional therapeutic benefit compared with established drugs. Several studies have revealed a beneficial effect of rolipram in inflammatory disease in vivo. In the rat model of experimental autoimmune encephalomyelitis, where TNF synthesis forms a central pathogenetic link, rolipram decreased disease activity (Sommer et al., 1995). In nonhuman primates, rolipram protected against autoimmune demyelinating disease even when administered after sensitization to central nervous system antigens (Genain et al., 1995). In rats, rolipram decreased clinical activity of experimental arthritis (Nyman et al., 1997; Ross et al., 1997). Additionally, LPS-induced TNF synthesis in mice could be suppressed by rolipram (Griswold et al., 1998). Rolipram reduced airway hyper-responsiveness in response to acute and chronic antigen exposure in monkeys (Turner et al., 1994). In this study, antigen-induced increases of TNF but not of IL-1 concentration were inhibited in bronchoalveolar lavage. This is in agreement with our in vitro findings of selective inhibition of TNF but not IL-1 synthesis by rolipram (Semmler et al., 1993).

Phosphodiesterase Inhibition and Inflammatory Bowel Disease. To date, specific type IV PDE inhibition has not been tested as a therapeutic strategy for inflammatory bowel disease. However, there are reports on the use of the nonspecific PDE inhibitor pentoxifylline for this indication. In the trinitrobenzene sulfonic acid model of colitis in rats, pentoxifylline treatment reduced the pathologic changes (Peterson and Davey, 1997). Yet, in a small clinical study with 16 patients with corticosteroid-dependent Crohn's disease, the administration of 400 mg of pentoxifylline administered four times a day did not improve clinical or histologic activity of disease (Bauditz et al., 1997). In vitro studies by the same research group revealed that a high concentration of pentoxifylline (IC_{50} = 25 μg/ml) was necessary to inhibit TNF synthesis in organ cultures of inflamed mucosa (Reimund et al., 1997). Nonspecific PDE inhibitors are less selective than rolipram and require a 500-fold higher concentration for inhibition of TNF synthesis. In human mononuclear cells, the concentration that inhibits TNF synthesis by 50% (IC_{50}) is 70 μM for pentoxifylline and 130 nM for rolipram (Semmler et al., 1993). This limits the therapeutic use of compounds such as pentoxifylline as anti-inflammatory agents. In the present study, we could demonstrate the suppression of colonic TNF concentrations by rolipram close to TNF concentrations observed in control mice. This emphasizes the mediatory function of TNF in this model.

The present study has some limitations. First, although DSS-induced colitis serves as a model for human disease, the cause of colitis in humans is not known and therefore other pathogenetic mechanisms may be at active. Second, due to the species studied and the particular situation of the animal model, we have examined a short disease course of 11 to 15 days, whereas inflammatory bowel disease in humans is chronic, extending over months and years. And third, i.p. administration is not a practicable route clinically and oral i.v. or topical formulations will have to be tested before the use in humans for this indication.

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References

Barnette MS, Bartus JO, Burman M, Christensen SB, Cieslinski LB, Esser KM, Prabhakar US, Rush JA and Torphy TJ (1996) Association of the anti-inflammatory activity of phosphodiesterase 4 (PDE4) inhibitors with either inhibition of PDE4 catalytic activity or competition for [3H]rolipram binding. *Biochem Pharmacol* **51**:949–956.

Bauditz J, Haemling J, Ortner M, Lochs H, Raedler A and Schreiber S (1997) Treatment with tumour necrosis factor inhibitor oxpentifylline does not improve corticosteroid dependent chronic active Crohn's disease. *Gut* **40**:470–494.

Beavo JA (1995) Cyclic nucleotide phosphodiesterases: Functional implications of multiple isoforms. *Physiol Rev* **75**:725–748.

Breese EJ, Michie CA, Nicholls SW, Murch SH, Williams CB, Domizio P, Walker-Smith JA and MacDonald TT (1994) Tumor necrosis factor α-producing cells in the intestinal mucosa of children with inflammatory bowel disease. *Gastroenterology* **106**:1455–1466.

Cooper HS, Murthy SN, Shah RS and Sedergran DJ (1993) Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest* **69**:238–249.

Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP and Elson CO (1994) Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* **107**:1643–1652.

Eigler A, Siegmund B, Emmerich U, Baumann KH, Hartmann G and Endres S (1998) Anti-inflammatory activities of cAMP-elevating agents: Enhancement of IL-10 synthesis and concurrent suppression of TNF production. *J Leukocyte Biol* **63**:101–107.

Eigler A, Sinha B, Hartmann G and Endres S (1997) Taming TNF: Strategies to restrain this proinflammatory cytokine. *Immunol Today* **18**:487–492.

Elliott MJ, Maini RN, Feldmann M, Kalden JR, Antoni C, Smolen JS, Leeb B, Breedveld FC, Macfarlane JD, Bijl H and Woody JN (1994) Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor-α (cA2) versus placebo in rheumatoid arthritis. *Lancet* **344**:1105–1110.

Elson CO, Sartor RB, Tennyson GS and Riddell RH (1995) Experimental models of inflammatory bowel disease. *Gastroenterology* **109**:1344–1367.

Essayan DM, Huang SK, Undem BJ, Kagey-Sobotka A and Lichtenstein LM (1994) Modulation of antigen- and mitogen-induced proliferative responses of peripheral blood mononuclear cells by nonselective and isozyme selective cyclic nucleotide phosphodiesterase inhibitors. *J Immunol* **153**:3408–3416.

Fekade D, Knox K, Hussein K, Melka A, Lalloo DG, Coxon RE and Warrell DA (1996) Prevention of Jarisch-Herxheimer reactions by treatment with antibodies against tumor necrosis factor alpha. *N Engl J Med* **335**:311–315.

Fischer W, Schudt C and Wendel A (1993) Protection by phosphodiesterase inhibitors against endotoxin-induced liver injury in galactosamine-sensitized mice. *Biochem Pharmacol* **45**:2399–2404.

Gantner F, Kusters S, Wendel A, Hatzelmann A, Schudt C and Tiegs G (1997) Protection from T cell-mediated murine liver failure by phosphodiesterase inhibitors. *J Pharmacol Exp Ther* **280**:53–60.

Genain CP, Roberts T, Davis RL, Nguyen MH, Uccelli A, Faulds D, Li Y, Hedgpeth J and Hauser SL (1995) Prevention of autoimmune demyelination in non-human primates by a cAMP-specific phosphodiesterase inhibitor. *Proc Natl Acad Sci USA* **92**:3601–3605.

Griswold DE, Webb EF, Badger AM, Gorycki PD, Levandoski PA, Barnette MA, Grous M, Christensens S and Torphy TJ (1998) SB 207499 (Ariflo), a second generation phosphodiesterase 4 inhibitor, reduces tumor necrosis factor-α and interleukin-4 production in vivo. *J Pharmacol Exp Ther* **287**:705–711.

Kim HS and Berstad A (1992) Experimental colitis in animal models. *Scand J Gastroenterol* **27**:529–537.

Kojouharoff G, Hans W, Obermeier F, Mannel DN, Andus T, Scholmerich J, Gross V and Falk W (1997) Neutralization of tumour necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. *Clin Exp Immunol* **107**:353–358.

Lehr HA, Mankoff DA, Corwin D, Santeusanio G and Gown AM (1997) Application of photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. *J Histochem Cytochem* **45**:1559–1565.

Murch SH, Braegger CP, Walker-Smith JA and MacDonald TT (1993) Location of tumour necrosis factor α by immunohistochemistry in chronic inflammatory bowel disease. *Gut* **34**:1705–1709.

Murch SH, Lamkin VA, Savage MO, Walker-Smith JA and MacDonald TT (1991) Serum concentrations of tumour necrosis factor-α in childhood chronic inflammatory bowel disease. *Gut* **32**:913–917.

Nyman U, Mussener A, Larsson E, Lorentzen J and Klareskog L (1997) Amelioration of collagen II-induced arthritis in rats by the type IV phosphodiesterase inhibitor Rolipram. *Clin Exp Immunol* **108**:415–419.

Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y and Nakaya R (1990) A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* **98**:694–702.

Olson AD, DelBuono EA, Bitar KN and Remick DG (1995) Antiserum to tumor necrosis factor and failure to prevent murine colitis. *J Pediatr Gastroenterol Nutr* **21**:410–418.

Peterson TC and Davey K (1997) Effect of acute pentoxifylline treatment in an experimental model of colitis. *Aliment Pharmacol Ther* **11**:575–580.

Prabhakar U, Lipshutz D, Bartus JO, Slivjak MJ, Smith EFr, Lee JC and Esser KM (1994) Characterization of cAMP-dependent inhibition of LPS-induced TNF-α production by rolipram, a specific phosphodiesterase IV (PDE IV) inhibitor. *Int J Immunopharmacol* **16**:805–816.

Present DH, Rutgeerts P, Targan S, Hanauer SB, Mayer L, Van Hozegand RA, Podolsky DK, Sands BE, Braakman T, DeWoody KL, Schaible TF and Van Deventer SJ (1999) Infliximab for the treatment of fistulas in patients with Crohn's disease. *N Engl J Med* **340**:1398–1405.

Reimund JM, Dumont S, Muller CD, Kenney JS, Kedinger M, Baumann R, Poindron P and Duclos B (1997) In vitro effects of oxpentifylline on inflammatory cytokine release in patients with inflammatory bowel disease. *Gut* **40**:475–480.

Ross SE, Williams RO, Mason LJ, Mauri C, Marinova-Mutafchieva L, Malfait AM, Maini RN and Feldmann M (1997) Suppression of TNF-α expression, inhibition of Th1 Activity, and amelioration of collagen-induced arthritis by rolipram. *J Immunol* **159**:6253–6259.

Sandborn WJ and Hanauer SB (1999) Antitumor necrosis factor therapy for inflammatory bowel disease: A review of agents, pharmacology, clinical results and safety. *Inflamm Bowel Dis* **5**:119–133.

Savendahl L, Underwood LE, Haldeman KM, Ulshen MH and Lund PK (1997) Fasting prevents experimental murine colitis produced by dextran sulfate sodium and decreases interleukin-1β and insulin-like growth factor I messenger ribonucleic acid. *Endocrinology* **138**:734–740.

Schade FU and Schudt C (1993) The specific type III and IV phosphodiesterase inhibitor zardaverine suppresses formation of tumor necrosis factor by macrophages. *Eur J Pharmacol* **230**:9–14.

Seldon PM, Barnes PJ, Meja K and Giembycz MA (1995) Suppression of lipopolysaccharide-induced tumor necrosis factor-α generation from human peripheral blood monocytes by inhibitors of phosphodiesterase 4: Interaction with stimulants of adenylyl cyclase. *Mol Pharmacol* **48**:747–457.

Semmler J, Wachtel H and Endres S (1993) The specific type IV phosphodiesterase inhibitor rolipram suppresses tumor necrosis factor-α production by human mononuclear cells. *Int J Immunopharmacol* **15**:409–413.

Siegmund B, Eigler A, Moeller J, Greten TF, Hartmann G and Endres S (1997) Suppression of tumor necrosis factor-α production by interleukin-10 is enhanced by cAMP-elevating agents. *Eur J Pharmacol* **321**:231–239.

Sinha B, Semmler J, Eisenhut T, Eigler A and Endres S (1995) Enhanced tumor necrosis factor suppression and cyclic adenosine monophosphate accumulation by combination of phosphodiesterase inhibitors and prostanoids. *Eur J Immunol* **25**:147–153.

Sommer N, Loschmann PA, Northoff GH, Weller M, Steinbrecher A, Steinbach JP, Lichtenfels R, Meyermann R, Riethmuller A, Fontana A, Dichgans J and Martin R (1995) The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. *Nat Med* **1**:244–248.

Souness JE, Houghton C, Sardar N and Withnall MT (1997) Evidence that cyclic AMP phosphodiesterase inhibitors suppress interleukin-2 release from murine splenocytes by interacting with a 'low-affinity' phosphodiesterase 4 conformer. *Br J Pharmacol* **121**:743–750.

Stack WA, Mann SD, Roy AJ, Heath P, Sopwith M, Freeman J, Holmes G, Long R, Forbes A, Kamm MA and Hawkey CJ (1997) Randomised controlled trial of CDP571 antibody to tumour necrosis factor-α in Crohn's disease. *Lancet* **349**:521–524.

Strieter RM, Remick DG, Ward PA, Spengler RN, Lynch JPd, Larrick J and Kunkel SL (1988) Cellular and molecular regulation of tumor necrosis factor-α production by pentoxifylline. *Biochem Biophys Res Commun* **155**:1230–1236.

Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, Schaible TF and Rutgeerts PJ (1997) A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor α for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* **337**:1029–1035.

Turner CR, Andresen CJ, Smith WB and Watson JW (1994) Effects of rolipram on responses to acute and chronic antigen exposure in monkeys. *Am J Respir Crit Care Med* **149**:1153–1159.

van Deventer SJ (1997) Tumour necrosis factor and Crohn's disease. *Gut* **40**:443–448.

van Dullemen HM, van Deventer SJ, Hommes DW, Bijl HA, Jansen J, Tytgat GN and Woody J (1995) Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology* **109**:129–135.

Wachtel H (1983) Potential antidepressant activity of rolipram and other selective cyclic adenosine 3′,5′-monophosphate phosphodiesterase inhibitors. *Neuropharmacology* **22**:267–272.

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Amelioration of collagen II-induced arthritis in rats by the type IV phosphodiesterase inhibitor Rolipram

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SUMMARY

The effect of Rolipram, a selective inhibitor of the cyclic AMP specific phosphodiesterase (PDE IV) was evaluated in the rat collagen type II (RCII)-induced arthritis model in the DA rat. Rolipram was given either shortly before expected onset of disease (days 10–14) or shortly after the onset of clinically evident arthritis (days 15–19 after immunization). Administration at days 10–14 delayed the onset of arthritis for approximately 5 days, but the severity of arthritis was thereafter comparable to that seen in a non-treated control group. Rolipram treatment of animals with manifest arthritis inhibited further arthritis development and also tended to diminish its severity at a phase of disease where non-treated control animals showed a rapidly progressing disease development. Serum levels of antibodies to RCII were in all experiments similar between Rolipram-treated and control animals. An *in situ* hybridization method for determining cytokine mRNA synthesis in regional lymph nodes, after administration of Rolipram (at days 2–7), demonstrated a strong inhibitory effect on tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) mRNA expression, whereas no effects were seen on IL-2 mRNA synthesis after *in vivo* challenge with native RCII emulsified in Freund's incomplete adjuvant. The results thus demonstrate strong preventive as well as therapeutic effects of Rolipram in a model for arthritis that is very similar to human rheumatoid arthritis with respect to cytokine regulation, and suggest that Rolipram has its major effects in the effector stage of the arthritogenic immune response.

Keywords arthritis collagen II Rolipram

INTRODUCTION

In trying to elucidate the value of new drug regimens in rheumatoid arthritis (RA), it is of critical importance to use animal models which share as many features as possible with the human disease, and in particular share the molecular mechanisms that are influenced by the drug in question.

The collagen-induced arthritis (CIA) which can be induced in mice as well as in rats [1–3] fulfils many of the requirements for similarities in clinical features and conventional histology; early symmetrical involvement of proximal interphalangeal joints as well as erosions of cartilage and bone are key characteristics of CIA. More recently, we have demonstrated striking similarities between the patterns of cytokine expression in the RA joint and in certain stages of CIA. In particular, there was in both cases a pronounced induction of tumour necrosis factor-alpha (TNF-α) synthesis in synovial cells within the pannus in close proximity to the cartilage. Induction of TNF-α synthesis in regional lymph nodes of collagen-immunized animals also appears to be a key feature in the induction of disease [4].

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The proinflammatory cytokines TNF-α and IFN-γ have emerged as key candidates for molecular targeting in the therapy of arthritis, not least from clinical demonstration of the efficacy of neutralizing anti-TNF-α antibodies in RA [5].

From a general point of view, a number of different TNF-α and IFN-γ inhibitors should be of interest for therapy of arthritis, and CIA should also be a good model to evaluate the value of such inhibitors, considering the apparent similarities between human RA and CIA in distribution and participation of TNF-α in the pathogenesis of these diseases. We also believe that the autologous CIA in the DA rat is a particularly relevant model in many of these contexts, as it typically shows a chronic and persistent course of disease.

Elevation of intracellular cAMP is a mechanism whereby TNF-α production is inhibited [6,7]. Both *in vivo* and *in vitro* this concept has been confirmed from results showing that the phosphodiesterase IV inhibitor Rolipram (4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone) [8] is effective in inhibiting the synthesis of TNF-α.

The marked inhibition of TNF-α expression by Rolipram may be explained by its specificity and high affinity to phosphodiesterase IV, which is the predominant isoenzyme in monocytes [9].

Rolipram is an interesting compound for several reasons. It was originally designed as an antidepressive drug, and clinical trials within this field have been performed. More recently, Rolipram has also been shown to ameliorate the development of experimental autoimmune encephalitis [10].

Against this background, we wanted to investigate the clinical effects of Rolipram in CIA in the DA rat, and at the same time analyse the effects of the drug on the immune response to collagen as well as the effect of the drug on local synthesis of TNF-α.

MATERIALS AND METHODS

Animals

Female DA rats, originally obtained from Zentralinstitut für Versuchstiersucht (Hannover, Germany) were kept and bred at the animal unit at the Karolinska Hospital (Stockholm, Sweden). The rats were kept in a separate animal room under climate-controlled conditions with a 12-h light/dark cycle, housed in polystyrene cages containing wood shavings and fed standard rodent chow and water *ad libitum*.

Immunization

DA rats were immunized intradermally, at the back of the tail, with 150 μg native rat collagen type II (RCII) prepared from chondrosarcoma, as described previously [11,12], dissolved in 0·1 M acetic acid and emulsified 1 : 1 with Freund's incomplete adjuvant (FIA; Difco, Detroit, MI).

Rolipram

Racemate was dissolved in physiological saline containing 100 g/l cremophor EL (Sigma, St Louis, MO) and given subcutaneously.

DA rats were injected with Rolipram twice daily: (i) four animals were injected with 2 mg/kg×2 at days 2–7 after immunization; (ii) eight animals were treated with 2 mg/kg×2 at days 10–14; (iii) eight animals were treated with 3 mg/kg×2, days 15–19.

Controls

The same number of DA rats as were treated with Rolipram only received physiological saline/cremophor mixture in the same administration regime as the treated animals.

Evaluation of arthritis

Arthritis develops normally in CII-immunized animals with 100% penetrance after about 2 weeks. Arthritis was assessed using a scale from 0 to 16, each of four paws being scored from 0 to 4, where 0 = no arthritis, 1–3 a summary of points as follows: swelling of the ankle, one point; swelling of one or more intratarsal and/or metatarsal joints, one point; swelling of one or more interphalangeal joints, one point; swelling of all joints, i.e. the entire paw, four points.

Quantification of humoral anti-collagen immunity

IgG antibody titres to native CII were determined using an ELISA, as previously described by Lorentzen *et al.* [13].

Cell preparation

Animals that received Rolipram between days 2 and 7 were killed 7 days after immunization with RCII/FIA. Individual lymph nodes were dissected out and transferred to a sterile tube with PBS. Single-cell suspensions were obtained by grinding the lymph nodes through a wire mesh in PBS. After washing in PBS×2, cells were loaded and centrifuged at 80*g* for 3 min in a cytocentrifuge. Thereafter, cells were fixed for 1 min in 4% paraformaldehyde in PBS at 4°C. Slides were stored in 70% ethanol in DEPC-treated distilled water at 4°C until assayed.

In situ *hybridization*

RNA probes for TNF-α, interferon-gamma (IFN-γ), IL-2 and IL-4, labelled by a α-^{32}S-UTP, were prepared by *in vitro* transcription of DNA templates, as described previously by Artursson *et al.* [14]. All experiments were performed with both antisense and sense RNA probes. The *in situ* hybridization was performed as described by Müssener *et al.* [4]. The results were expressed as number of labelled cells per $1{\cdot}0\times10^5$ cells.

RESULTS

The effects of prophylactic administration of Rolipram are demonstrated in Fig. 1. The graphs illustrate the clinical outcome, measured as arthritis score, in DA rats treated with subcutaneous injections of Rolipram, 2 mg/kg, or control vehicle twice daily for 5 days, between days 10 and 14 after immunization with FIA and RCII. The given mean score at each time point is the aritmetric mean score within the group. The animals in the Rolipram-treated group were refractory to development of arthritis at the presumed day of onset (about day 14). However, some 5 days after expected onset and after cessation of Rolipram administration, these animals started to develop an arthritis that demonstrated all the characteristics of arthritis in the untreated animals and reached the same top score as the control group. The animals in the control group demonstrated an arthritis development comparable to animals not receiving any type of treatment after immunization.

The results of treatment of manifest arthritis with Rolipram are demonstrated in Fig. 2. The graphs demonstrate the effects on arthritis scores in DA rats treated with subcutaneous injection of Rolipram, 3 mg/kg, or control vehicle, twice daily, between days 15 and 19 after immunization with FIA and RCII. Thus, treatment was initiated at a time point when arthritis was apparent. The given value at each time point is the average score within the group and corresponds to the evaluation system used in Fig. 1. The development of arthritis among the animals in the Rolipram-treated group changed drastically compared with animals in the control group. Progression of arthritis severity was halted, and after 5 days of treatment there was even a tendency towards decreased arthritis scores in the Rolipram-treated group. Between 2 and 6 days after cessation of Rolipram treatment, arthritis scores increased but did not reach the same level as in the untreated arthritis case during the observation period.

Normally the humoral immune response after immunization with RCII/FIA demonstrates a rising titre of antibodies against RCII during the development of arthritis. In the present investigation all animals developed antibodies aginst RCII. No differences in serum levels of anti-CII antibodies, as measured with an ELISA assay, could be demonstrated between any of the groups of Rolipram-treated animals or animals in the two control groups.

The effects of Rolipram treatment, 2 mg/kg, between days 2 and 7, on the expression of TNF-α, IFN-γ, IL-2 and IL-4 mRNA in regional lymph nodes were analysed. This time in the investigation was chosen on the basis of previous experiments, where significant expression in regional lymph nodes of TNF-α was demonstrated 7 days after immunization with CII [4]. Animals that received Rolipram between days 2 and 7 after immunization with FIA and

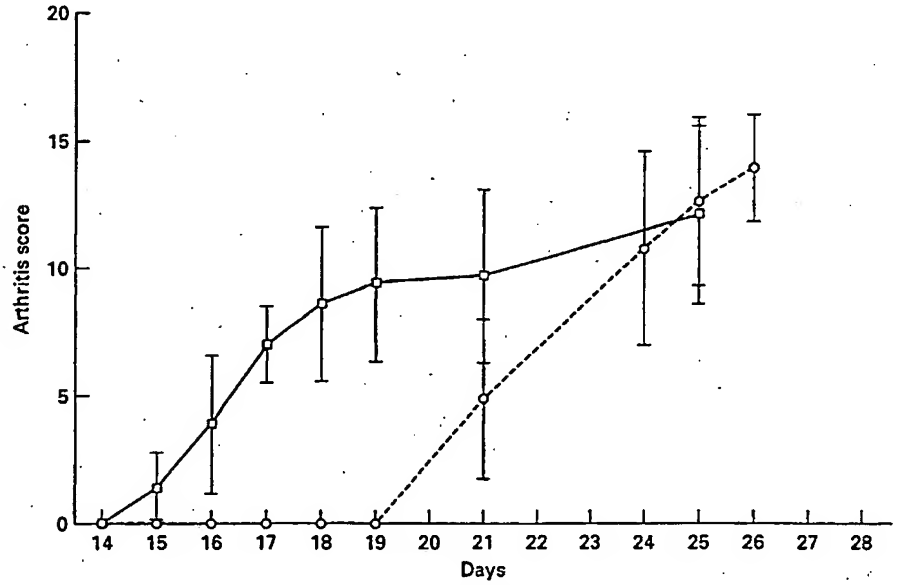

Fig. 1. The effects on arthritis scores in DA rats treated with subcutaneous injection of Rolipram (○; 2 mg/kg×2) or a mixture of physiological saline and Cremophor (control, □) between days 10 and 14 after immunization with Freund's incomplete adjuvant and collagen II, but before onset of manifest disease. Rolipram-treated animals were refractory to development of arthritis at the presumed day of onset. However, some 5 days after expected onset, these animals started to develop an arthritis that demonstrated all the characteristics of arthritis in untreated animals, and reached the same arthritis top score as the control group. Mean values are shown and bars indicate 1 s.d.

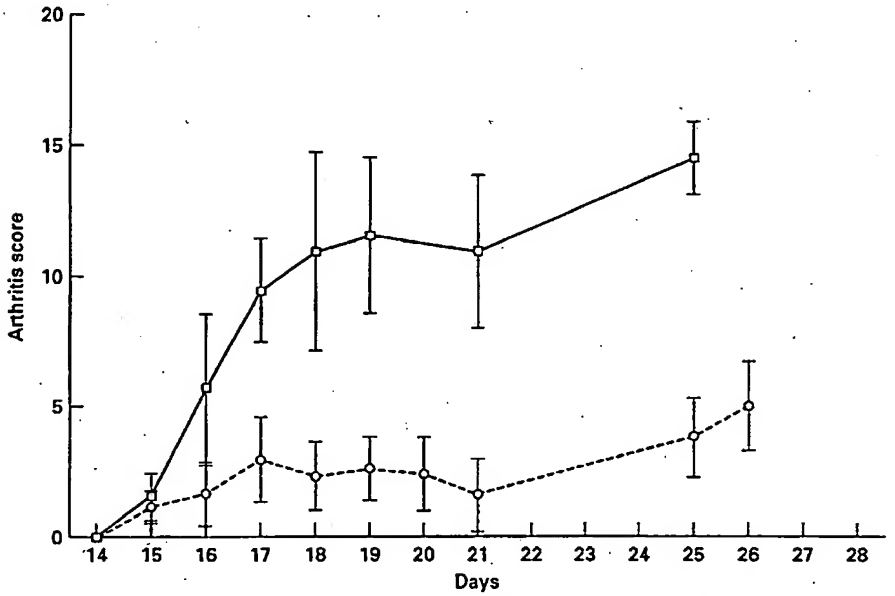

Fig. 2. The effects on arthritis scores in DA rats treated with subcutaneous injection of Rolipram (○; 3 mg/kg×2) or a mixture of physiological saline and Cremophor (control, □) between days 15 and 19 after immunization with Freund's incomplete adjuvant and collagen II. Treatment was initiated at a time point when arthritis was apparent. The development of arthritis in the Rolipram-treated group changed drastically compared with animals in the control group. Progression of severity was halted and after 5 days of treatment there was a tendency towards decreased arthritis scores. Between 2 and 6 days after cessation of treatment the summarized arthritis score increased again, but did not reach the levels observed in animals with untreated arthritis. Mean values are shown and bars indicate 1 s.d.

Table 1. Effect of Rolipram compared with control on expression of cytokine mRNA in cells isolated from draining lymph nodes of DA rats 7 days after immunization with rat collagen type II (RCII) in mineral oil

	TNF-α	IL-2	IFN-γ	IL-4
RCII	71 (± 7)	13 (± 7)	8 (± 2)	0
RCII + Rolipram	10 (± 4)	12 (± 6)	0	0

The results are expressed as mean numbers of tumour necrosis factor-alpha (TNF-α), IL-2, IFN-γ and IL-4 producing cells per $1{\cdot}0 \times 10^5$ lymph node cells ± s.d. (As demonstrated in the Table, a strong inhibitory effect of Rolipram was observed on TNF-α mRNA and IFN-γ mRNA synthesis, whereas no effects were seen on IL-2 mRNA synthesis. IL-4 mRNA expression could not be demonstrated in the lymph nodes of treated animals or controls.)

RCII were killed after the last day of treatment. Regional draining lymph nodes were isolated and cells prepared for *in situ* hybridization. The results of the present *in situ* hybridization are demonstrated in Table 1, and are expressed as numbers of labelled cells per 10^5 cells. As demonstrated in the Table, a strong inhibitory effect of Rolipram was observed on the expression of TNF-α and IFN-γ mRNA, whereas no effect was seen on IL-2 mRNA synthesis. IL-4 mRNA expression could not be demonstrated in the lymph nodes of either treated or control animals.

DISCUSSION

The pivotal role of TNF-α in the development of arthritis has been recognized for some time, and much effort has been focused on various means of interfering with the expression of this monokine in order to influence the course of arthritis. Previously reported results on Rolipram, a selective inhibitor of the cyclic AMP specific phosphodiesterase IV (PDE IV), with a documented down-regulating effect on TNF-α *in vitro*, initiated the present study. The effect of Rolipram was evaluated in the CIA model in the DA rat, a model that resembles RA and typically shows a chronic and persistent course of disease.

A substantial clinical effect on the development of arthritis was evident after administration of Rolipram. Also, expression of TNF-α and IFN-γ mRNA in regional draining lymph nodes was strongly down-regulated.

In order to distinguish between potentially therapeutic and prophylactic properties of Rolipram, the compound was given to different groups of animals either before or after onset of arthritis. Onset of arthritis could be delayed as long as treatment with Rolipram was continued, indicating a prophylactic potential. However, after cessation of drug administration clinical arthritis developed comparable to that seen in untreated control groups.

On the other hand, Rolipram treatment of animals with manifest arthritis inhibited further aggravation of the arthritis, and also tended to diminish its severity at a phase of disease where untreated control animals showed a rapidly progressing disease development.

We were also able to demonstrate a very strong influence by Rolipram on one of the earliest, and probably very important, events in the development of arthritis, the strong expression of TNF-α and IFN-γ mRNA in regional lymph nodes. This effect on cytokine expression correlated well with the observed clinical effects on arthritis. The expression of both these cytokines was strongly down-regulated by Rolipram, while the expression of IL-2 was unaffected. The pronounced effect on the expression of IFN-γ was somewhat unexpected, since earlier *in vitro* studies indicated a more modest impact on this cytokine.

In comparison with previous studies, especially those showing the beneficial effects of treatment with MoAbs against TNF-α in both rodent arthritis models [5] and human RA [15], Rolipram in the investigated model seems to be at least as efficient.

The presented data are also in accordance with the findings of other groups working in the field of experimental allergic encephalomyelitis (EAE) models. Sommer *et al.* [10] showed that rats treated several times a day with subcutaneous injections of Rolipram were protected from developing EAE. It was also quite recently demonstrated by Genain [16] that monkeys receiving 10 mg/kg of Rolipram every other day were protected from development of clinical EAE. Interestingly, the Rolipram-treated animals as well as control animals were developing circulating antibodies to myelin basic protein, but treated animals developed no MRI abnormalities. Sekut *et al.* [17] also reported on the anti-inflammatory activity of Rolipram in several murine models, among them the carrageenan-induced paw oedema model and the adjuvant arthritis model, where administration of Rolipram reduced local inflammation.

We could not demonstrate that Rolipram had any immunomodulating properties in this experimental setting. In all experiments, serum levels of antibodies against RCII were comparable in treated animals and in control groups. Inflammatory arthritis as well as other organ-specific autoimmune diseases are believed to be mediated by IFN-γ-producing Th1 cells. In the reported experiments, expression of IFN-γ mRNA was decreased by Rolipram, but no IL-4 was produced. The findings thus indicate that the major effect of Rolipram is exerted in the effector phase of the inflammatory process.

Also, the observation that arthritis regains aggressiveness after cessation of Rolipram treatment indicates that in order to achieve an immunomodulating effect, or a permanent down-regulation of the arthritis, Rolipram treatment must be combined with a tolerance- or anergy-inducing regime.

In conclusion, the results demonstrate strong preventive as well as therapeutic effects of Rolipram in a model for arthritis that is very similar to human RA with respect to cytokine regulation, and suggest that Rolipram has its major effects in the effector stage of the arthritogenic response.

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REFERENCES

1 Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen: an experimental model of arthritis. J Exp Med 1977; 146:857–68.

2 Courtenay JS, Dallman MJ, Dayan AD *et al.* Immunization against heterologous type II collagen induces arthritis in mice. Nature 1980; 283:666–8.

3 Holmdahl R, Jansson L, Gullberg D *et al.* Incidence of arthritis and autoreactivity of anti-collagen antibodies after immunization of heterologous and autologous collagen II. Clin Exp Immunol 1985; 62:639–46.

4 Müssener Å, Klareskog L, Lorentzen J, Kleinau S. TNF-alpha dominates cytokine mRNA expression in lymphoid tissues of rats developing collagen- and oil-induced arthritis. Scand J Immunol 1995; 42:128–34.
5 Elliot MJ, Maini RM, Feldmann M *et al.* Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis. Lancet 1994; 344:1105–10.
6 Taffet SM, Singhel KJ, Overholtzer JF, Shurtleff SA. Regulation of tumor necrosis factor expression in a macrophage like cell line by lipopolysaccharide and cyclic AMP. Cell Immunol 1989; 120:291–300.
7 Endres S, Fülle HJ, Sinha B *et al.* Cyclic nucleotides differentially regulate the synthesis of tumor necrosis factor alpha and interleukin 1β by human mononuclear cells. Immunology 1991; 72:56–60.
8 Semmler J, Wachtel H, Endres S. The specific type IV phosphodiesterase inhibitor Rolipram suppresses tumor necrosis factor alpha production by human mononuclear cells. Int J Immunopharmacol 1993; 15:3:409–13.
9 Tannenbaum CS, Hamilton TA. Lipopolysaccharide-induced gene exprerssion in murine peritoneal macrophages is selectively suppressed by agents that elevate intracellular cAMP. J Immunol 1989; 142:1274–80.
10 Sommer N, Löschmann PA, Northoff GH *et al.* The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. Nature Med 1995; 1:244–8.
11 Smith BD, Martin GR, Miller EJ, Dorfman A, Swarm R. Nature of the collagen synthesized by a transplanted chondrosarcoma. Arch Biochem Biophys 1975; 166:181–6.
12 Andersson ME, Holmdahl R. Analysis of type II collagen reactive T cells in the mouse. Different regulation of autoreactive vs. non-autoreactive anti-type II collagen T cells in the DBA/1 mouse. Eur J Immunol 1990; 20:1061–6.
13 Lorentzen JC, Erlandsson H, Müssener Å *et al.* Specific and long-lasting protection from collagen-induced arthritis and oil-induced arthritis in DA rats by administration of immunogens. Scand J Immunol 1995; 42:82–89.
14 Artursson K, Gobl A, Lindersson M, Johansson M, Alm G. Molecular cloning of gene encoding porcrine IFN-β. J Interferon Res 1992; 12:153–60.
15 Williams RO, Feldman M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci USA 1992; 89:9784–8.
16 Genain CP. Prevention of autoimmune demyelination in non-human primates by a c-AMP-specific phosphodiesterase inhibitor. PNAS USA 1995; 92:3601–5.
17 Sekut L, Yarnell D, Stimpson SA *et al.* Anti-inflammatory activity of phosphodiesterase (PDE)-IV inhibitors in acute and chronic models of inflammation. Clin Exp Immunol 1995; 100:126–32.

Type 4 Phosphodiesterase Inhibitors Have Clinical and *In Vitro* Anti-inflammatory Effects in Atopic Dermatitis

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Increased cyclic AMP-phosphodiesterase activity in peripheral blood leukocytes is associated with the immune and inflammatory hyperreactivity that characterizes atopic dermatitis. Atopic phosphodiesterase has high sensitivity to a variety of enzyme inhibitors, suggesting an increased therapeutic advantage. The objective of this study was to use *in vitro* assays to identify a potent phosphodiesterase inhibitor and then to investigate its effectiveness in treating atopic dermatitis.

Leukocyte enzyme activity was measured by radioenzyme assay, whereas prostaglandin E_2 and interleukins 10 (IL-10) and 4 (IL-4) were measured in 24-h culture supernatants of mononuclear leukocytes by immunoassays. The effect of a topical phosphodiesterase inhibitor on atopic dermatitis lesional skin was assessed by double-blind, paired comparisons of active drug and placebo ointments applied to symmetrically involved sites over a 28-d period.

Using *in vitro* assays, we demonstrated the ability of selective high-potency phosphodiesterase inhibitors to reduce prostaglandin E_2, IL-10, and IL-4 production in atopic mononuclear leukocyte cultures. We selected the Type 4 phosphodiesterase inhibitor, CP80,633, based on its inhibitory potency, for clinical testing by topical, bilateral paired comparisons in 20 patients with atopic dermatitis and demonstrated significant reductions of all inflammatory parameters.

Phosphodiesterase inhibitors modulate several pathways contributing to the exaggerated immune and inflammatory responses, which characterize atopic dermatitis. This *in vivo* demonstration of anti-inflammatory efficacy may provide a useful alternative to the over-reliance on corticosteroid therapy in atopic disease. *Key words: PDE/IL-4/IL-10/monocytes. J Invest Dermatol 107:51–56, 1996*

Cyclic nucleotide phosphodiesterases comprise a family of isoenzymes that hydrolyze the 3′,5′-cyclic nucleotides to 5′-nucleotide monophosphates (Beavo, 1988). We have been particularly interested in cAMP phosphodiesterase (PDE), because of the increased cAMP hydrolytic activity in leukocytes from patients with atopic dermatitis (AD), asthma, and allergic rhinitis (Grewe *et al*, 1982). These diseases represent a symptom complex characterized by immunologic hyper-reactivity and by inappropriate inflammatory cell infiltration into skin and respiratory tissues. The abnormal PDE activity correlates with leukocyte functional defects including basophil histamine hyper-releasibility (Butler *et al*, 1983) and increased B-lymphocyte IgE production (Cooper *et al*, 1985), both of which are normalized by *in vitro* incubation with the PDE inhibitor (PDE-i), Ro 20-1724.

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Abbreviations: PDE, phosphodiesterase; AD, atopic dermatitis; PDE-i, phosphodiesterase inhibitor; MNL, mononuclear leukocytes; FBS, fetal bovine serum; IFN-γ, interferon-γ; Th1, Type 1 T helper cells; Th2, Type 2 T helper cells; ELISA, enzyme-linked immunosorbent assay.

Phosphodiesterases have in recent years been classified into seven families (Types I–VII or, by genome terminology, PDE1–7) according to a number of characteristics including sensitivity to inhibitors (Beavo, 1990). In previous studies, we found evidence that the more active PDE4 in atopic leukocytes had increased sensitivity to inhibition by Ro 20-1724 and other agents (Giustina *et al*, 1984; Chan and Hanifin, 1993), compared to PDE in normal leukocytes. We have utilized this technique to assay the potency of PDE4 inhibitors, comparing effects on PDE activity in atopic and normal mononuclear leukocytes (MNL) (Chan and Hanifin, 1993). In this study, we conducted an *in vitro* survey of several compounds shown to be potent PDE4 inhibitors. Among these, we found that two enantiomers, CP80,633 (Cohan *et al*, 1995) and CP102,995, and the racemate, CP76,593, had the highest potency in comparison to other agents. Consistent with past studies (Giustina *et al*, 1984; Chan and Hanifin, 1993), these compounds showed a greater relative specificity for the atopic compared to the normal PDE isoenzyme. These techniques appear to provide a relevant *in vitro* system for predicting the therapeutic efficacy of each new PDE-i in the management of AD, asthma, and other inflammatory diseases. Focusing on the higher potency inhibitors of PDE, we assayed the effectiveness of new compounds on eicosanoid and cytokine production *in vitro*. We then carried out a double-blind, vehicle-controlled, paired-comparison study to assess the safety and efficacy

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of one of the new compounds when applied topically for treatment of AD. Our studies demonstrate that these PDE inhibitors modulate multiple immune and inflammatory pathways and significantly reduce signs and symptoms of atopic inflammation.

MATERIALS AND METHODS

RPMI-1640 medium, Gey's balanced salt solution, fetal bovine serum (FBS), neuraminidase, Hanks' balanced salt solution, Hanks' calcium/magnesium-free balanced salt solution: Gibco (Grand Island, NY). 5'-Nucleotidase, cAMP, imidazole, and snake venom (5'-nucleotidase): Sigma Chemical Co., St. Louis, MO. Hypaque-Ficoll: Pharmacia, Piscataway, NJ. [^{3}H]cAMP (36 Ci/mmol): New England Nuclear, Boston, MA. Ion exchange resin AG1X2 (200–400 mesh): Bio-Rad, Richmond, CA. "Ready-Solv" scintillation fluid: Beckman, San Jose, CA. Anti-CD3 (OKT3): Ortho Diagnostics, Rahway, NJ. Ro 20-1724 was a gift from Hoffmann LaRoche, Nutley, NJ. CP76,593 and its resolved enantiomers, CP80,633 (Cohan *et al*, 1995) and CP102,995, were received from Central Research Division, Pfizer Inc., Groton, CT.

Subjects All subjects gave informed consent approved by the institutional Human Research Committee. For leukocyte studies, venous blood was drawn at 8:00 a.m., and immediately mixed with heparin (10 units/ml) for further processing. Normal, healthy subjects had no personal history of asthma, allergic rhinitis, or AD. Patients with active AD were chosen according to well-defined criteria (Hanifin and Rajka, 1980) and blood donors had moderate to severe disease. Blood donors' ages ranged from 25 to 52 years (9 males and 11 females) for normal subjects and 20 to 50 years for AD subjects (10 males and 8 females). Individual experiments were not precisely age- and gender-matched, because leukocytes were used in various assays on any given day. Subjects for the clinical trial had lesions not exceeding 20% of total body surface area. No donors had received antihistamine or topical corticosteroid therapy for at least 96 h prior to study, and none had used systemic adrenergic, PDE inhibitors, or corticosteroid medications for at least 1 mo. No caffeine or other methylxanthine-containing beverages were consumed within 14 h prior to leukocyte studies.

Cell Preparations Blood was separated by Hypaque-Ficoll gradient centrifugation at 400 × *g* for 30 min, and MNL were harvested from the interphase of plasma and separation fluid (Chan and Hanifin, 1993). The cells were washed three times with saline and spun at 400, 300, and 250 × *g* sequentially to eliminate platelet contamination. MNL were harvested and counted using a Coulter counter. Differential-lymphocyte and -monocyte quantitations utilized Giemsa and acid naphthyl acetate esterase stains and latex bead phagocytic ingestion. These quantitations were monitored in all preparations and showed no differences between AD and normal subjects in terms of percentages of monocytes and lymphocytes. MNL were either freeze-thawed three times in an acetone-dry ice bath, and the homogenates were stored at −80°C until assayed for homogenate PDE activity, or cultured as described below. To obtain monocytes, MNL at 4 × 10^6 cells/ml were allowed to adhere in a 16- × 100-mm petri dish for at least 2 h at 37°C in RPMI-1640 + 10% FBS. The nonadherent cells were decanted and washed three times with warm Gey's balanced salt solution. The adherent monocytes were recovered by scraping with a sterile rubber policeman. The MNL compositions, determined by Stat Stain (Volu-Sol; Logos Scientific, Henderson, NV), contained 10–40% monocytes, 60–70% lymphocytes, ≤2% polymorphonuclear leukocytes, and ≤5% platelets. The nonadherent cells were typically ≥95% $CD3^+$ lymphocytes. Cell viability, monitored by trypan blue exclusion test, was always >98%. Monocyte purity in the adherent cell preparations, confirmed by acid naphthyl acetate esterase and Factor XIII immunoperoxidase staining, was ≥95%.

For PGE_2 production, monocytes (2 × 10^6/ml) were incubated in RPMI-1640/10% FBS. For IL-4 production, MNL (2 × 10^6/ml) were incubated in RPMI-1640/10% FBS with 10 ng/ml anti-CD3 (Chan *et al*, 1993). After 24 h, supernatants were harvested by pelleting cells at 700 × *g*.

PDE Inhibition/Assay Homogenized leukocyte preparations were kept at 4°C, and various PDE inhibitors were immediately added. All inhibitors were dissolved in 50% ethanol at a concentration of 10^{-2} M, then further diluted in Gey's balanced salt solution to appropriate concentrations. For the determination of IC_{50}, 10^{-8} to 10^{-4} M final concentrations of inhibitors or control buffer were used. The mixtures were then incubated at 37°C for 60 min. PDE activities were determined in the presence of the inhibitors or in control buffers in all experiments. Ro 20-1724 was used as a reference compound for comparison of inhibitor effects in all studies. Maximum inhibition for each compound was determined by curve-fitting with a computer program, and the concentration giving 50% of maximum inhibition was recorded as IC_{50}.

PDE was assayed using a modified method of Thompson *et al* (1979). The incubation mixture (0.4 ml) contained 1 μM cAMP, 200,000 cpm of [^{3}H]cAMP, and 0.2 ml of sample (10^7 cells/ml) or standard PDE in 40 mM Tris-Cl buffer (pH 8.0) containing 3.75 mM β-mercaptoethanol and 15 mM $MgCl_2$. After incubation at 30°C for 10 min, the reaction was terminated by snap-freezing in ethanol-dry ice bath and the mixture was then boiled for 1 min. Purified 5'-nucleotidase was added to the mixtures, which were then further incubated for 10 min at 30°C and then transferred to Pasteur pipette columns containing ion exchange resin AG1X2 to remove the remaining nucleotides and nucleosides. The radioactivity in the eluates was quantitated in scintillation fluid. Enzyme activity was expressed as picomoles of cAMP hydrolyzed per μg of protein. Protein concentration was determined by an assay using Bio-Rad protein dye. A standard PDE from bovine heart was used to monitor consistency and recovery in each assay (Chan and Hanifin, 1993).

Immunoassays Prostaglandin E_2 (PGE_2) was assayed by radioimmunoassay as previously described (Chan *et al*, 1993), using culture supernatants containing 0.05 mM indomethacin as blank. PGE_2 antisera were produced in rabbits according to the method of Jaffe and Behrman (1974). PGE_2 was conjugated to porcine thyroglobulin by the mixed anhydride method before immunization of the rabbits. At a dilution of 1:6000, the PGE_2 antiserum had a sensitivity of 10 pg/0.1 ml of sample and the following cross-reactivities at B/B_0 50% were: PGD_2, 0.8%; $PGF_{2\alpha}$, 1%; $PGF_{1\alpha}$, 0.3%; PGE_1, 9.3%; 6-keto-$PGF_{1\alpha}$, 2.2%; and 6-keto-PGE_2, 2%, as previously reported (Geissler *et al*, 1989).

Quantitative determination of human IL-4 was performed using enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN) with modification to increase sensitivity (Li *et al*, 1993). The practical sensitivity of the assay was 5 pg/ml. In order to measure below 5 pg/ml, the ELISA procedure was modified, according to manufacturer's instructions, by extending the color development time at low concentrations from 15 to 45 min to generate a linear scale between 0 and 5 pg/ml. A human IL-10 ELISA kit was also used to determine IL-10 concentrations. The lower limit of detection was 7.8 pg/ml for undiluted monocyte culture supernatants. PDE inhibitors did not interfere with the IL-10 standard curves.

Topical Therapy Twenty patients with AD (12 males and 8 females) aged 18 to 50 years were enrolled in the study to determine efficacy of topical CP80,633 ointment for treatment of skin inflammation. Exclusions included females with childbearing potential, pregnant or nursing women, and patients who required any medication that might interact with or obscure treatment effects including the use of oral theophylline derivatives, oral, parenteral, or topically applied corticosteroids, and H1 or H2 antihistamines. Patients had to be in good health with normal laboratory parameters and electrocardiograms.

The study design was a right/left paired-comparison study to compare the efficacy of topically applied CP80,633 (0.5%) ointment twice daily for 28 d with its petrolatum vehicle on 200-cm^2 lesional areas. Active drug and vehicle were assigned by side in a randomized, double-blind fashion. Patients were selected for the presence of symmetrically involved anatomical sites on the right and left sides excluding the hands, feet, and face. Grading each of three inflammatory parameters [(i) erythema; (ii) induration/papulation; (iii) excoriation] utilized a scale from 0 to 3 (1 = mild, 2 = moderate, 3 = severe, with half steps) and baseline scores were required to be at least 6 of the possible 9 for the total clinical score. The subjective itch score was likewise graded on a scale of 0–3. After physical examination, blood chemistries [alkaline phosphatase, alanine and aspartate aminotransferase (ALT and AST), Na^+, K^+, Cl^-, glucose, uric acid, blood urea nitrogen, creatinine, and total bilirubin], urinalysis and hematology (complete blood count with differential) were obtained from venous blood. Study drug was applied twice daily for 28 d of treatment. Overall efficacy assessments were made at days 3, 7, 14, 21, and 28 to grade improvement or worsening of inflammatory signs, determined by comparing to baseline scores the specific parameters and the total clinical score. Repeat laboratory evaluations were performed at days 7, 14, and 28 or at the time of early discontinuation. Electrocardiogram was repeated at the end of study.

Statistical Analysis For *in vitro* studies comparing IC_{50} values of PDE and IL-4 production in cultures, Student's t test was used, whereas Mann-Whitney nonparametric analysis was used in comparing effects of PDE inhibitors on PGE_2 and IL-10 production. For clinical studies, individual parameter scores and the sum of scores were compared for active and placebo-treated sites. The score at each time point was subtracted from the baseline score and this change was analyzed using two-sided t tests,

Table I. Comparison of 50% Inhibition Concentrations (IC_{50}) Against Phosphodiesterase Activity in Homogenates of Mononuclear Leukocytes from Patients with Atopic Dermatitis (AD) and from Normal Subjects[a]

Phosphodiesterase Inhibitors	IC_{50} (μM, mean ± SEM)		
	AD (n)[b]	Normal (n)	Values[c]
Pentoxyfylline	1.76 ± 0.26 (4)	3.58 ± 1.22 (3)	0.045
Rolipram	0.86 ± 0.22 (7)	1.13 ± 1.86 (7)	0.037
Theophylline	27.11 ± 12.43 (7)	87.92 ± 19.68 (9)	0.027
Ro 20-1724	0.17 ± 0.08 (8)	2.9 ± 0.55 (8)	0.0076
CP76,593	0.32 ± 0.12 (11)	4.43 ± 1.60 (5)	0.00176
CP80,633	0.015 ± 0.003 (10)	ND	
CP102,995	0.88 ± 0.007 (8)	ND	

[a] Ficoll-Hypaque gradient-separated peripheral blood mononuclear leukocytes were homogenized and incubated with final concentrations of each inhibitor ranged from 10^{-8} to 10^{-4}M to determine their IC_{50} for the inhibition of PDE activity.
[b] (n) = no. of donors; ND = not done.
[c] p values were determined by Student's t test comparing IC_{50} between normal and AD.

employing a level of significance ($p < 0.05$) to test the alternative hypothesis that the mean paired difference is not equal to zero.

RESULTS

Greater Type 4 Inhibition of Atopic PDE Our first objective was to compare potencies of newer Type 4 inhibitors with presently available agents on PDE activity in MNL homogenates. We initially determined mean IC_{50} values of PDE inhibition in MNL homogenates by the racemic mixtures CP76,593, for both normal and AD groups **(Table I)**, and compared these mean IC_{50} values from AD and normal groups with those of pentoxyfylline, rolipram, theophylline, and Ro 20-1724. Results showed that CP76,593, which was slightly less potent than Ro 20-1724, was more active against the AD isozyme than the other inhibitors. Each of these compounds had less potency against the normal isozyme, consistent with our previous findings (Giustina *et al*, 1984; Chan and Hanifin, 1993).

We then compared pure enantiomers (+) CP80,633 and (−) CP102,995, with (±) CP76,593 and found that CP80,633 was 21- and 59-fold more inhibitory than either CP76,593 ($p < 0.01$) or CP102,995 ($p = 0.008$), respectively, against the PDE in MNL from patients with AD **(Table I)**. CP80,633 was also significantly more active than Ro 20-1724 ($p < 0.001$). Because the focus of our studies was AD inflammation, pure enantiomers were not tested on normal cells.

PDE Inhibitors Reduce Atopic Monocyte PGE_2 and IL-10 Production We recently reported increased spontaneous PGE_2 (Chan *et al*, 1993) and IL-10 (Ohmen *et al*, 1995) production by cultured monocytes from patients with AD. We found that these increases corresponded with elevated PDE activity and hypothesize that this results in inadequate cAMP modulation of monocyte function (Chan *et al*, 1993). To evaluate the effect of PDE inhibitors, PGE_2 levels were measured by radioimmunoassay in 24-h culture supernatants from monocytes treated with CP80,633 (0.1 μM) or Ro 20-1724 (1 μM), comparing AD and normal preparations. The concentrations of the inhibitors used were chosen for their respective maximal effects, as determined by dose-response curves. Untreated control cultures confirmed previous findings of markedly elevated mean PGE_2 levels in AD preparations (562 ± 107 *versus* 89 ± 27 for normals, mean ± SEM $p = 0.0036$; **Fig 1**). Both CP80,633 and Ro 20-1724 caused significant reductions in PGE_2 supernatant levels in AD compared to the untreated control culture ($n = 4–8$). Ro 20-1724 did not affect PGE_2 production in normal cell cultures. This was consistent with the reduced enzyme inhibition in normal cells **(Table I)** and with previous findings of increased inhibitor sensitivity of atopic PDE (Giustina *et al*, 1984; Chan and Hanifin, 1993). The PGE_2 changes

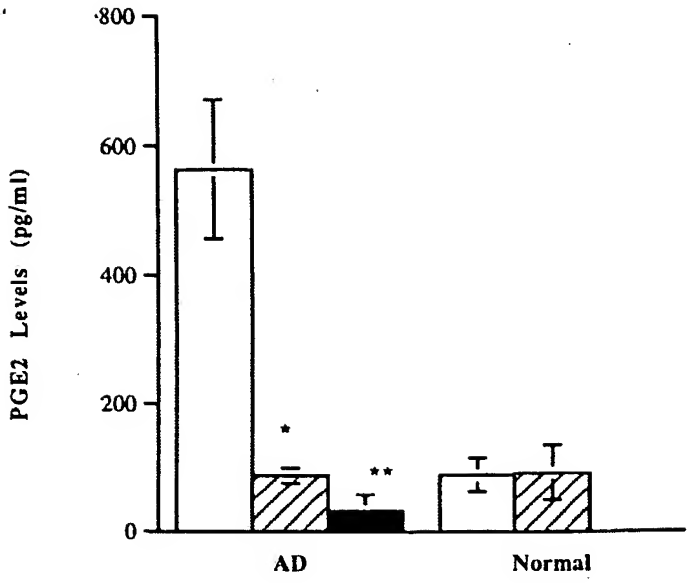

Figure 1. Prostaglandin E_2 levels in monocyte culture supernatants. Prostaglandin E_2 (PGE_2) levels (pg/ml, mean ± SEM) in unstimulated culture supernatants from atopic dermatitis (AD) and normal monocytes cultured for 24 h with media alone (□), Ro 20-1724 (1 μM, ▨), or CP80,633 (0.1 μM, ■; not done in normals). *$p = 0.004$. †$p < 0.001$.

in Ro 20-1724 and CP80,633-treated AD cell cultures were significant ($p < 0.001$ and < 0.001, respectively) by Mann-Whitney.

We also compared Ro 20-1724 and CP80,633 inhibition of AD monocyte IL-10 production in a dose-response experiment **(Fig 2)**. The concentration of CP80,633 required for 50% inhibition of IL-10 (IC_{50}) was 2.2 nM, indicating a 1000-fold greater potency than Ro 20-1724 ($IC_{50} = 2.5$ μM) in reducing production of this cytokine ($p < 0.001$, by Mann-Whitney test). Initial experiments using the usual 10^{-7} M CP80,633 concentration showed 94% and 100% inhibition of spontaneous IL-10 production in normal ($n = 2$) and AD ($n = 4$) monocyte cultures, respectively. Mean spontaneous IL-10 production in these studies by normal monocytes was 578 ± 118 pg/ml ($n = 2$) and 1962 ± 276 pg/ml ($n = 5$) by AD monocytes.

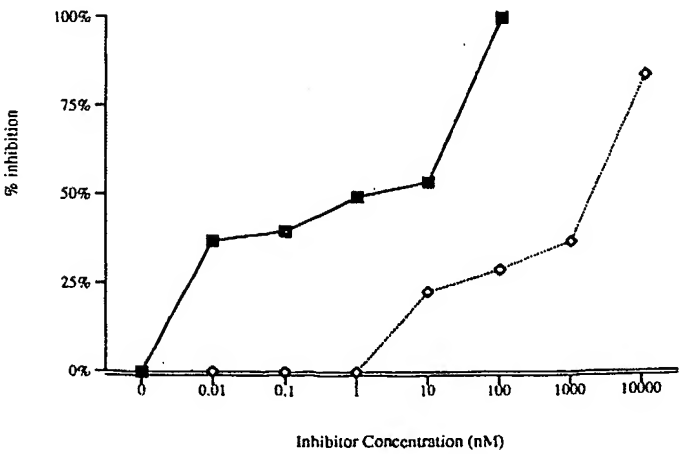

Figure 2. Dose effect of PDE inhibitors on monocyte IL-10 production. Dose-related inhibition of spontaneous atopic monocyte IL-10 production by Ro 20-1724 (◇) and CP80,633 (■). Spontaneous IL-10 production for this donor was 2330 pg/ml.

Table II. PDE Inhibitor Effects on IL-4 Production[a]

	AD (n)	p value[b]	Normal (n)	p value[b]
Control	42.6 ± 6.7 (8)		17.6 ± 1.9 (6)	
+ Ro 20-1724	5.2 ± 2.1 (6)	0.003	14.2 ± 1.6 (6)	NS[c]
+ CP80,633	2.4 ± 2.5 (3)	0.036	21.9 ± 4.6 (3)	NS

[a] Mononuclear leukocytes were cultured for 24 h with anti-CD3 (10 ng/ml) ± Ro 20-1724 (1 μM) or CP80,633 (0.1 μM). Supernatant IL-4 levels were measured by ELISA and expressed as pg/ml mean ± SEM.
[b] p value comparing inhibitor effect to controls..
[c] NS = not significant.

Reduced IL-4 Production We previously noted an inverse relationship between elevated PDE activity and reduced interferon-γ (IFN-γ) levels in atopic MNL (Chan *et al*, 1993) and reasoned that increased IL-4 production might likewise relate to abnormal PDE activity. We assessed the effect of PDE inhibitors on IL-4 production determined by ELISA from supernatants of anti-CD3–stimulated AD and normal 24-h MNL cultures, with and without PDE inhibitors. As can be seen in **Table II**, both Ro 20-1724 (1 μM) and CP80,633 (0.1 μM) caused 8- and 18-fold reductions, respectively, in AD supernatant concentrations. These inhibitors had no effect on normal IL-4 supernatant levels.

Topical CP80,633 as an Anti-Inflammatory Agent Based on its demonstrated PDE inhibitory potency, CP80,633 was selected for clinical testing. Twenty patients with AD were enrolled in a clinical trial to assess the efficacy and safety of CP80,633. Symmetrical, moderately involved areas of up to 200 cm^2 on each of the right and left sides were selected for assessment of active *versus* placebo therapy. The baseline mean total scores at bilateral sites were comparable (5.20 ± 0.22 *versus* 5.33 ± 0.19, $p = 0.27$). Efficacy evaluations were recorded at days 3, 7, 14, 21, and 28 during therapy. Inflammation was quantitated by the same observer grading erythema, induration/papulation, and excoriation on a scale from 0 to 3 (none, mild, moderate, or severe). Baseline scores compared with those at day 28 showed significant improvement on sites receiving CP80,633 with mean reductions in erythema ($p = 0.004$), induration ($p < 0.001$), and excoriations ($p = 0.046$), the latter serving as an objective indicator of pruritus. Patients were also asked to estimate the level of itching at each visit, and their subjective responses likewise showed significant improvement on active compared to placebo-treated sites ($p = 0.002$). The response to active drug was consistent among the subjects, with an improvement in total clinical score observed in 16 of 20 CP80,633-treated sites (**Fig 3**), graded at the last assessment, compared to only three of 20 placebo-treated sites (mean ± SD 1.40 ± 1.76 and −0.65 ± 1.48, respectively; $p < 0.001$).

Figure 4 shows the mean change from baseline of the total clinical scores (erythema + induration + excoriation) of active and placebo-treated sites over the course of the study. Mean baseline scores were similar for vehicle and CP80,633-treated sites. Significantly reduced inflammation was evident as early as day 3 and continued throughout the therapy phase for actively treated sites, which demonstrated significantly greater improvement than placebo sites at each time point. Because we had previously detected *in vitro* evidence of tachyphylaxis among asthmatic patients treated with theophylline long-term (Giustina *et al*, 1984) we were interested in whether this might occur with topical CP80,633 therapy; however, improvement continued throughout the 4-wk course of treatment with active drug.

Adverse events indistinguishable from manifestations of AD (itching, burning, folliculitis) were noted as single events on nine placebo- and nine vehicle-treated sites, and 16 of these 18 observations were bilateral. In two instances, folliculitis was noted on the active drug-treated site and in two other patients, bilateral folliculitis was noted. These events are typical of AD (Hanifin and Rajka, 1980) and the occurrence of folliculitis in 5.3% of active site

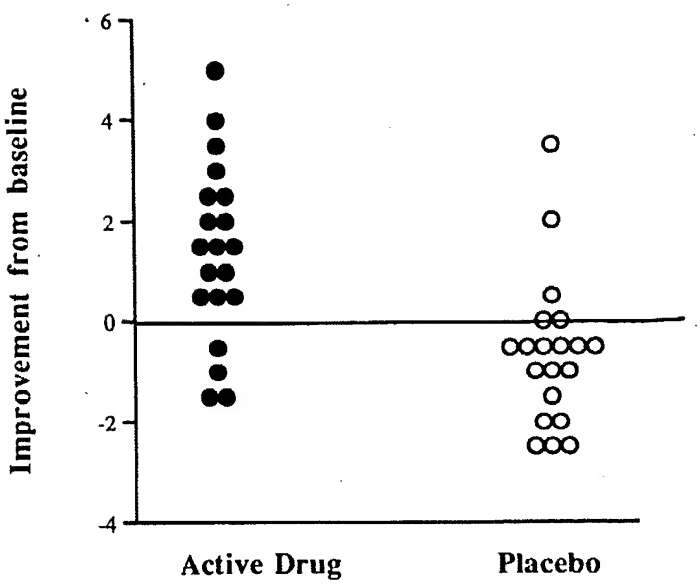

Figure 3. Clinical effect of topical PDE inhibitor, CP80,633. Clinical effect (change in mean total score of clinical parameters) of topical phosphodiesterase inhibitor, CP80,633, on bilateral atopic dermatitis lesions. Each point represents the difference between total clinical score at baseline and the score at the last observed assessment.

observations was not significantly greater than the 2.6% frequency on vehicle-treated sites. There were no drop-outs for adverse events and no clinically relevant laboratory or electrocardiogram changes. Eight patients discontinued before the end of the treatment period, all due to flaring of dermatitis on untreated areas (no other therapy was allowed during the course of the trial). In three of the eight patients, dermatitis became intolerable on one side, the placebo-treated side; in the other five patients, the study was

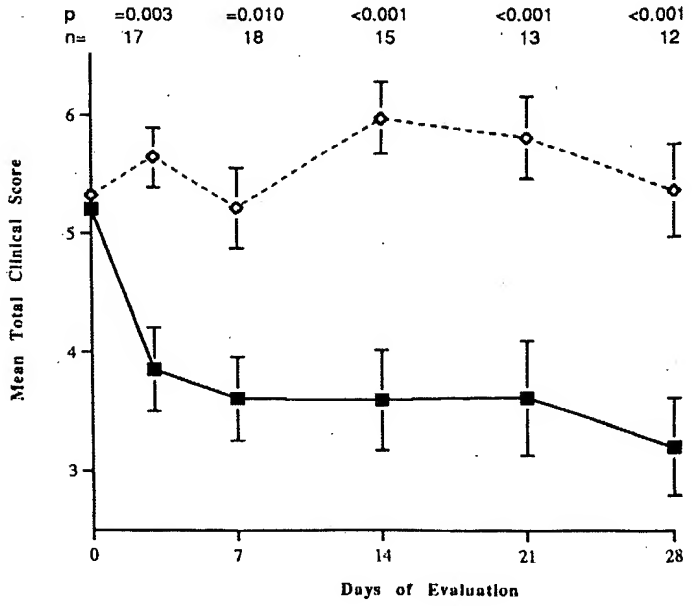

Figure 4. Time-course of change in clinical scores for active *versus* placebo-treated atopic dermatitis lesions. Longitudinal pattern of clinical response reflected by mean change (± SEM) from baseline total clinical scores of topical CP80,633 (■) *versus* placebo-treated atopic dermatitis (◇) sites. Significant improvement was evident at each time point (*p* values indicated); n = number of subjects evaluated at each time point during therapy (days 3–28).

discontinued because of generalized worsening of the untreated dermatitis.

DISCUSSION

Atopic dermatitis, a chronic inflammatory skin disease, causes severe pruritus leading to excoriation and secondary infection. Economically, AD creates a considerable health care burden (Lapidus *et al*, 1993), accounting for 1% of pediatric outpatient visits (Sampson, 1990) and, because of lifelong cutaneous hyperirritability, over 80% of occupational skin disease (Shmunes and Keil, 1983; Nassif *et al*, 1994). Therapeutic options for AD, as for allergic respiratory disease, are limited and inadequate. Glucocorticosteroids are used almost exclusively, but toxic effects are evident in many patients. For some very severe cases, photochemotherapy, cyclosporin A, or IFN-γ are used, but these are high-risk, expensive, and generally unsatisfactory modalities. Safe, effective anti-inflammatory agents for treatment of AD are perhaps the greatest need and challenge in dermatologic disease.

Altered cyclic nucleotide metabolism in atopic disease was predicted previously by Szentivanyi (1968). This led to a series of studies that demonstrated blunting of cAMP responses in leukocytes of patients with AD. We showed that this defect was caused by high cAMP hydrolysis by PDE in atopic leukocytes (Grewe *et al*, 1982; Butler *et al*, 1983). The increased PDE activity was present even in cord blood cells of newborns from atopic parents (Heskel *et al*, 1984), indicating an intrinsic, possibly genetically controlled abnormality. We demonstrated that the increased PDE correlated with histamine hyper-releasibility and with elevated spontaneous IgE production in cultured AD leukocytes, and we showed that PDE inhibitors could normalize those functions (Butler *et al*, 1983; Cooper *et al*, 1985).

Other studies demonstrated that the atopic isoenzymes were distinctly more sensitive to each PDE-i (Giustina *et al*, 1984; Chan and Hanifin, 1993), suggesting that these agents have a therapeutic advantage in AD. Our studies have focused particularly on blood monocytes which have a major proportion of abnormal PDE activity. We recently presented evidence that AD monocytes also have a considerable immune modulating effect on T cells. IFN-γ production, which is reduced in MNL cultures, became normal or elevated in purified T-cell cultures, indicating a monocyte inhibitory effect on Type 1 T helper cells (Th1) (Chan *et al*, 1993). This led to the demonstration of increased spontaneous production of PGE_2 (Chan *et al*, 1993) and IL-10 (Ohmen *et al*, 1995), both known suppressors of IFN-γ production by Th1 cells.

These studies strongly suggest that increased PDE activity reduces intracellular cAMP levels that, in turn, allow greater basal monocyte secretion of T-cell modulators. Because of the association of increased PDE activity with the elevated PGE_2 and IL-10 production in AD monocytes, we reasoned that each PDE-i might correct these abnormalities. Comparisons in enzyme inhibition assays **(Table I)** showed that a new agent, CP80,633, was 10-fold more potent than the standard Type 4 inhibitor, Ro 20-1724. We found that CP80,633 had a greater inhibitory effect on PGE_2 and IL-10 production by AD monocytes and on IL-4 production in cultures of MNL. We cannot clearly state whether the IL-4 effect was indirect, by inhibiting monocyte modulating factors, or occurred directly on Type 2 T helper (Th2) cells. It could also be a combined effect, because we have observed PDE inhibition in lymphocytes, though this action occurred with a specific Type III inhibitor, nitraquazone (Chan and Hanifin, 1993), and CP80,633 is not an inhibitor of Type III PDE (Cohan *et al*, 1995).

It seems reasonable to consider that abnormal PDE isoenzymes underlie many of the immune and inflammatory abnormalities of atopic disease. We have demonstrated that inhibition of PDE influences a number of cellular and mediator pathways including monocyte PGE_2 and IL-10 synthesis, and IL-4 over-production. Because of CP80,633's effects in enzyme and cytokine inhibition and other predictive assays (Cohan *et al*, 1995), as well as the distinctly higher sensitivity of AD enzyme to PDE inhibitors, we tested the drug in clinical studies, comparing active 0.5% CP80,633 with placebo ointment vehicle applied to symmetrical right and left lesions in 20 patients with AD. Responses were prompt, showing statistically significant improvement within 3 d and maintaining throughout the 28-d trial. The drug reduced inflammation in 80% (16 of 20) treated sites, as compared to only 3 of 20 placebo sites. Importantly, no irritation or other adverse events were observed in a disease notoriously subject to irritancy.

Topical treatment is the preferred method for most patients with AD and is an area of great need because of the common involvement of face and eyelids, thin-skinned areas in which corticosteroids may cause atrophy. Systemic use of PDE inhibitors has been limited by side effects, particularly the common nausea and vomiting resulting from use of high-dose theophylline, and a particular problem with newer, more potent agents (Torphy and Undem, 1991); however, this high potency compound, CP80,633, clearly reduces atopic inflammation when applied topically on the skin. To date, we have noted no evidence of emesis or other side effects with topical use, though considerable absorption might be expected if large areas of skin were treated. Our study suggests that drugs of this type may also have potential for treating asthma via the inhalant topical route.

The clinical anti-inflammatory effectiveness by a potent Type 4 PDE-i applied to the skin confirms predictions from *in vitro* studies. This class of drugs, by increasing intracellular cAMP levels and reducing cytokine and mediator release, modulates exaggerated atopic responses by multiple immune and inflammatory cells. Single pathway inhibitors may be inadequate for controlling the many facets of inflammatory responses. It is hoped that this *in vivo* demonstration of efficacy will encourage development of useful alternatives to replace over-reliance on toxic corticosteroids in atopic disease.

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REFERENCES

Beavo JA: Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv Cyc Nuc Res* 22:1–38, 1988

Beavo JA: In: Beavo J, Houslay MD (eds.). *Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action. Multiple Phosphodiesterase Isoenzymes: Background, Nomenclature and Implications*. John Wiley & Son, New York, 1990, pp 3–15

Butler JM, Chan SC, Stevens SR, Hanifin JM: Increased leukocyte histamine release with elevated cyclic AMP-phosphodiesterase activity in atopic dermatitis. *J Allergy Clin Immunol* 71:490–497, 1983

Chan SC, Hanifin JM: Differential inhibitor effects on cyclic AMP phosphodiesterase isoforms in atopic and normal leukocytes. *J Lab Clin Med* 121:44–51, 1993

Chan SC, Kim J-W, Henderson WR, Hanifin JM: Altered prostaglandin E_2 regulation of cytokine production in atopic dermatitis. *J Immunol* 151:3345–3352, 1993

Cohan VL, Showell HJ, Pettipher ER, Fisher DA, Pazoles CJ, Watson JW, Turner CR, Cheng JB: *In vitro* pharmacology of the novel type IV phosphodiesterase (PDE_{IV}) inhibitor, CP80,633. *J Allergy Clin Immunol* 95:350, 1995

Cooper KD, Kang KF, Chan SC, Hanifin JM: Phosphodiesterase inhibition by Ro 20-1724 reduces hyper-IgE synthesis by atopic dermatitis cells *in vitro*. *J Invest Dermatol* 84:477–482, 1985

Geissler FT, Kuzan FB, Faustman EM, Henderson WR, Jr.: Lipid mediator production by post-implantation rat embryos *in vitro*. *Prostaglandins* 38:145–155, 1989

Giustina TA, Chan SC, Baker JW, Hanifin JM: Increased leukocyte sensitivity to PDE inhibitors in atopic dermatitis: tachyphylaxis after theophylline therapy. *J Allergy Clin Immunol* 74:252–257, 1984

Grewe S, Chan SC, Hanifin JM: Elevated leukocyte cyclic AMP-phosphodiesterase in atopic disease: a possible mechanism for cyclic AMP-agonist hyporesponsiveness. *J Allergy Clin Immunol* 70:452–457, 1982

Hanifin JM, Rajka G: Diagnostic features of atopic dermatitis. *Acta Derm Venereol Suppl* 92:44–47, 1980

Heskel NS, Chan SC, Thiel ML, Stevens SR, Casperson LS, Hanifin JM: Elevated umbilical cord blood leukocyte cyclic adenosine monophosphate-phosphodiesterase activity in children with atopic parents. *J Am Acad Dermatol* 11:422–426, 1984

Jaffe BM, Behrman HR (eds.): *Hormone Radioimmunoassay. Prostaglandins E, A, and F*. Academic Press, New York, 1974, p 19

Lapidus CS, Schwarz DF, Honig PJ: Atopic dermatitis in children: Who cares? Who pays? *J Am Acad Dermatol* 28:699–703, 1993

Li SH, Chan SC, Toshitani A, Leung DYM, Hanifin JM: Synergistic effects of

interleukin 4 and interferon-gamma on monocyte phosphodiesterase activity. *J Invest Dermatol* 99:65–70, 1993

Nassif A, Chan SC, Storrs FJ, and Hanifin JM: Abnormal skin irritancy in atopic dermatitis and in atopy without dermatitis. *Arch Dermatol* 130:1402–1407, 1994

Ohmen JD, Hanifin JM, Nickoloff BJ, Rea TH, Wyzykowski R, Kim J, Jullien D, McHugh T, Nassif AS, Chan SC, Modlin RL: Overexpression of IL-10 in atopic dermatitis: contrasting cytokine patterns with delayed-type hypersensitivity reactions. *J Immunol* 154:1956–1963, 1995

Sampson HA: Pathogenesis of eczema. *Clin Exp Allergy* 1990;20:459

Shmunes E, Keil JE: Occupational dermatoses in South Carolina: a descriptive analysis of cost variables. *J Am Acad Dermatol* 9:861–866, 1983

Szentivanyi A: The beta-adrenergic theory of the atopic abnormality in bronchial asthma. *J Allergy* 42:203–232, 1968

Thompson WJ, Terasaki WL, Epstein PM, Strada SJ: Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular form of the enzyme. *Adv Cyclic Nucleotide Res* 10:69–92, 1979

Torphy TJ, Undem BJ: Phosphodiesterase inhibitors: new opportunities for the treatment of asthma. *Thorax* 46:512–523, 1991

0022-202X/79/7304-0261$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 73:261-263, 1979

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REPORTS

Ro 20-1724: An Agent that Significantly Improves Psoriatic Lesions in Double-Blind Clinical Trials

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Two double-blind studies comparing the effectiveness of the cyclic nucleotide-altering agent (4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone) (Ro 20-1724) vs vehicle have demonstrated that this compound can improve psoriatic lesions. Although Ro 20-1724 was not as effective as intensive occlusive treatment of psoriatic lesions with 0.025% triamcinolone acetonide, Ro 20-1724 had no adverse systemic or cutaneous effects. Ro 20-1724 and other cyclic nucleotide-altering agents may have therapeutic potential in the future treatment of psoriasis.

Psoriasis is the prototype of several common steroid sensitive proliferative skin diseases and afflicts approximately 1 to 2% of the population [1]. Although the precise role of cyclic nucleotides in psoriasis remains unclear [2-6], most investigators do agree that the epidermal cyclic nucleotide metabolism in psoriatic plaques may be misregulated.

Recent investigations [7] with the cyclic nucleotide phosphodiesterase (PDE) inhibitors papaverine and Ro 20-1724 (4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone) have shown that papaverine inhibits both cAMP-PDE and cGMP-PDE, whereas Ro 20-1724 selectively inhibits only cAMP-PDE. Furthermore, when psoriatic epidermis was incubated with papaverine or Ro 20-1724, Ro 20-1724 increased the levels of cAMP significantly greater than papaverine incubations. These studies clearly demonstrated the superiority of Ro 20-1724 to selectively elevate cAMP levels in incubated psoriatic epidermis.

Since the topical application of 1% papaverine cream produces a modest but significant improvement of psoriatic lesions [8], we anticipated that the marked *in vitro* superiority of Ro 20-1724 to elevate cyclic AMP levels and its lipophilic character would also result in the improvement of psoriatic lesions. Therefore, we investigated the clinical effectiveness of Ro 20-1724 in 3 double-blind clinical studies with psoriatic patients. In these studies, we compared the clinical effectiveness of Ro 20-1724 to vehicle and to 0.025% triamcinolone acetonide.

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Abbreviations:
cAMP: 3',5'-cyclic adenosine monophosphate
cGMP: 3',5'-cyclic guanosine monophosphate
PDE: phosphodiesterase
Ro 20-1724: 4(3-butoxy-4-methoxybenzyl-2-imidazolidinone)

MATERIALS AND METHODS

Comparison of 1% Ro 20-1724 Cream and its Vehicle

Seventeen outpatients with typical plaques of psoriasis, who had not treated the disease for one week, participated in a double-blind clinical study. Two lesions approximately 5 cm in diameter were selected on each patient and designated as site 1 and site 2.

Patients were 18 yr of age or older, with no history of significant cardiovascular disease. Women chosen were postmenopausal, had had a hysterectomy or were on oral-contraceptives. Patients being treated with insulin or oral hypoglycemics were not accepted to the study. For this clinical evaluation the creams were supplied by Hoffman-LaRoche, Inc., Nutley, New Jersey. The vehicle cream contained: glyceryl monostereate, cetyl alcohol, white petrolatum, methylparaben, prophyparaben, propylene glycol, and purified water. The Ro 20-1724 formulation consisted of the same cream with Ro 20-1724 added to a 1% final concentration. Each patient received vehicle cream and Ro 20 cream in tube containers. One of each pair of tubes had been randomly designated for use on site 1 and the other for use on site 2. Both creams had the same color. The code was not broken until completion of the study.

Patients applied the creams 4 times a day for 4 weeks under occlusion. The total occlusion was approximately 20 hr per day. Topical steroids and systemic medication for psoriasis were not permitted to be used concurrently. Clinical laboratory studies were conducted on all patients before using the creams and at the first 2 subsequent visits. The laboratory parameters obtained included electrolytes, urea nitrogen, total bilirubin, glucose, total protein, albumin, cholesterol, calcium, phosphorus, uric acid, creatinine, glutamic oxaloacetic transaminase, lactic dehydrogenase, alkaline phosphatase, white blood count, hematocrit, hemoglobin, red blood count and urine analysis for sugar, ketones and proteins.

Each test lesion was evaluated independently by 2 physicians, 2, 4 and 6 weeks after the initiation of the study. The final evaluation at 6 weeks was made following a 2-week period with no application. Improvement was judged by a decrease in lesion thickness, not by a loss of scale or change in color. Each lesion was assigned a score of 0 on the first day and improvement from the initial status was scored as follows: 0 = no improvement or worsening, 1 = minimal improvement, 2 = moderate improvement (decreased thickness and/or incomplete clearing), 3 = complete clearing with or without residual redness or change in pigmentation. When 2 physicians disagreed in their evaluation, the lower score was used for statistical analysis. Scores of 2 and 3 were called improved, and scores of 0 and 1 were called unimproved in the analysis of the results at either 2, 4 or six weeks of application.

Patients showing differential improvement, i.e., who had a lesion respond to Ro 20 while the matched lesion did not respond to a vehicle, or who had a lesion respond to vehicle while the matched lesion did not respond to Ro 20, were used for statistical evaluations employing a sign test [9].

Comparison of 3 Ro 20-1724 Concentrations at the University of Miami

A second study comparing Ro 20 at concentrations of 0.25%, 0.5%, and 1% vs vehicle cream was conducted at the University of Miami by one of us (G.W.) in part to look for a dose response effect. Nine patients

were treated simultaneously with three Ro 20 concentrations and vehicle cream in a double-blind psoriasis therapeutic study. Patients did not receive any systemic or topical antipsoriatic medications during the study. On multiple similar psoriatic plaques, 10 sites were delineated approximately 1-½" square which were randomly assigned to be treated with the different Ro 20 concentrations and vehicle cream. Approximately 0.5 ml of each concentration of Ro 20 and vehicle were applied in duplicate to the appropriate sites. The remaining 2 sites were untreated controls. Creams were applied daily under occlusion for 6 days, treatment was stopped on the 7th day, then applications were resumed for an additional 6 days. All test applications in the study were made by a technician to insure the greatest accuracy in the drug being applied each time to the same site. Each site was scored as 0 if there was no change from initial status, 1+ if there was minimal improvement, and 2+ if there was substantial improvement or clearing. Daily evaluations were recorded after the treated sites had been washed with water and acetone. The final evaluation was made on the 15th day, 2 days after the final treatment.

Comparison of 1% Ro 20-1724 Cream and 0.025% Triamcinolone Acetonide Cream

Thirty-three outpatients participated in a third study which compared the effect of 1% Ro 20-1724 vs 0.025% triamcinolone (TMC). The patients and psoriatic lesions were selected in the identical manner to the first study. The TMC was in a vehicle which contained: sorbic acid, potassium sorbate, mono- and diglycerides, squalane, polysorbate 80 USP, stearyl alcohol USP, polysorbate 60, spermaceti USP, and sorbitol solution USP in a water base. This vehicle and that for Ro 20 were identical in color and odor. The Ro 20 formulation was identical to that used in the first clinical study. The methods of application and evaluation from the first study were employed (see "Comparison of 1% Ro 20-1724 Cream and its Vehicle").

RESULTS AND DISCUSSION

Twelve of 17 patients completed the first clinical study. Two patients were disqualified because they did not occlude the lesions regularly, and 3 requested to be removed because their untreated psoriasis was flaring. The clinical laboratory studies did not reveal any abnormalities which could be attributed to 1% Ro 20-1724.

Table I summarizes the data obtained from the 4th week evaluation of those 12 patients. Nine of 12, 75%, of the patients improved only with the Ro 20 preparation and 8% (1 of 12) improved only with the vehicle. The Ro 20 treated site was improved in 9 of 10 patients showing differential improvement ($p = .011$).

The results of this study can be compared with the results of the previously reported papaverine vs vehicle psoriasis bioassay [8]. Utilizing the percent relative difference ± SE [10], the proportion of patients expected to improve with 1% Ro 20 or 1% PAP cream who did not improve with the respective vehicle creams can be determined. This comparison shows that Ro 20 is a much superior agent in treating psoriasis since 73 ± 15% of patients would be expected to improve with 1% Ro 20 cream in contrast to only 39 ± 12% receiving 1% PAP cream ($p = .05$).

Nine psoriasis patients completed the second (University of Miami) clinical study. All 54 sites treated with the 3 concentrations of Ro 20-1724 showed either minimal or substantial improvement (Table II). The placebo preparation had only 4 minimial responses out of 18 test sites suggesting a definite therapeutic effect from the Ro 20-1724 preparations ($p < .01$). A dose related effect is suggested at the 0.25% concentration, however, this concentration may also be too high to demonstrate this point.

Thirty of thirty-three patients completed the third clinical study. Two patients were dropped from the study because they did not apply the creams regularly and the other patient was disqualified because he did not occlude the lesions. None of the clinical laboratory studies revealed any abnormalities attributable to 1% Ro 20-1724.

The results of the second and the fourth week evaluation of those 30 patients are summarized in Table III. Every patient whose Ro 20-treated lesion improved by the 4th week also improved on TMC. The difference in the time course of improvement for TMC and Ro 20 can be seen in Table IV. Eighty-six percent of the patients had improved on TMC at the second week and the increase to 94% at the fourth week was not significant ($p = .25$). By contrast, the percentage for Ro increased from 43 to 77 which is significant ($p < .001$). Ro 20 and TMC appeared to be clinically similar.

Eight of the nine patients judged completely cleared at the 4th week on Ro 20 were seen 2 weeks after the applications were discontinued. Six of 8 (75%) had begun to relapse. Twenty of 23 patients who cleared completely on TMC were evaluated after two weeks without application. Eight of these (40%) partially relapsed.

These investigations indicate that Ro 20-1724 is capable of improving psoriasis. In 2 double-blind studies comparing Ro 20-1724 vs vehicle, Ro 20-1724 significantly improved psoriasis in geographically distinct (Michigan and Florida) patient populations. Although Ro 20-1724 is not as effective as 0.025% triamcinolone acetonide applied q.i.d. under occlusion, no atrophy, striae or other adverse steroid effects would be expected with Ro 20-1724 application.

The currently available therapeutic modalities to treat psoriasis have inherent limitations that restrict their effectiveness. Of those most commonly employed, topical steroids can induce atrophy and striae and UVB irradiation with topical coal tar usually requires hospitalization. Furthermore, the risk factors

TABLE I. *Percent of 12 psoriasis patients with matched lesions responding to 1% Ro 20-1724 vs vehicle at 4 weeks."*

	Percent
Improved on *both* Ro 20-1724 and vehicle	0
Improved on Ro 20-1724 *only*	75*
Improved on vehicle *only*	8*
Unimproved on *both*	17
	100
Sign test (one-sided hypothesis)	* $p = .011$

" Categorized into 4 possible patterns of response (improved on *both*, improved on Ro 20 *only*, improved on vehicle *only*, unimproved on *both*)

TABLE II. *Clinical effects by drug concentration of Ro 20-1724 on psoriasis"*

Concentrations of Ro 20-1724	Number of test sites at each concentration				
	1%	.5%	.25%	Placebo	
	Response				
	2	11	11	8	0
	1	7	7	10	4
	0	0	0	0	14

" Each of the nine patients tested in duplicate sites with the preparations above (see text). A possible dose response effect may be present at the 0.25% concentration. Response was graded as follows: no improvement = 0, minimal improvement = 1, and moderate or better improvement = 2.

TABLE III. *Percent of 30 psoriasis patients with matched lesions responding to 1% Ro 20-1724 vs 0.025% triamcinolone categorized into the 4 possible patterns of response at 2 & 4 weeks"*

	Percent	
	2nd Week	4th Week
Improved on *both* Ro 20-1724 and Triamcinolone	43	77
Improved on Ro 20-1724 *only*	0*	0†
Improved on Triamcinolone *only*	43*	17†
Unimproved on *both*	14	6
	100	100
Sign test (one-sided hypothesis)	* $p < .001$	† $p = .03$

" Improved on *both*, improved on Ro 20 *only*, *improved on TMC only*, unimproved on *both*.

TABLE IV. *Percent of lesions improved in 30 psoriasis patients on .025% triamcinolone and 1% Ro 20-1724 at 2 & 4 weeks*

	Percent	
	2nd Week	4th Week**
Triamcinolone-treated	86°	94°
Ro 20-1724-treated	43†	77†

Sign test (one-sided hypothesis) * $p = .25$, † $p < .001$.
** All lesions improved at 2nd week were also improved at 4th week.

of PUVA cannot be objectively assessed at this time. Ro 20-1724 and other cyclic nucleotide-altering agents are experimental agents that may have future potential in the treatment of psoriasis. Such agents may provide topical therapy with a minimum of adverse side effects which can be used as alternative forms of treatment for patients with psoriasis who are unable to use conventional forms of therapy.

REFERENCES

1. Van Scott EJ, Farber EM: Dermatology in General Medicine. Edited by TB Fitzpatrick, KA Arndt, WH Clark, Jr., AC Eisen, EJ Van Scott, JH Vaughan. New York, McGraw-Hill, 1971, p 219.
2. Voorhees JJ, Duell EA, Bass LJ, Harrell ER: Decreased cyclic AMP in the epidermis of lesions of psoriasis. Arch Dermatol 105695–701, 1972
3. Voorhees JJ, Stawiski MA, Duell EA, Haddox MK, Goldberg ND: Increased cyclic GMP and decreased cyclic AMP levels in the hyperplastic abnormally differentiated epidermis of psoriasis. Life Sci 13:639–653, 1973
4. Harkonen M, Hopsu-Havu VK, Raij K: Cyclic adenosine monophosphate, adenyl cyclase, and cyclic nucleotide phosphodiesterase in psoriatic epidermis. Acta Dermatovener (Stockh) 54:13–18, 1974
5. Yoshikawa K, Adachi K, Levine V, Halprin KM: Micro-determination of cyclic AMP levels in human epidermis, dermis and hair follicles. Br J Dermatol 92:241–248, 1975
6. Halprin KM, Adachi K, Yoshikawa K, Levine V, Mai MM, Hsia SL: Cyclic AMP and psoriasis. J Invest Dermatol 65:170–178, 1975
7. Rusin LJ, Duell EA, Voorhees JJ: Papaverine and Ro 20-1724 inhibit cyclic nucleotide phosphodiesterase activity and increase cyclic AMP levels in psoriatic epidermis in vitro. J Invest Dermatol 71:154–156, 1978
8. Stawiski MA, Powell JA, Lang PG, Schork MA, Duell EA, Voorhees JJ: Papaverine: Its effects on cyclic AMP *in vitro* and psoriasis *in vitro*. J Invest Dermatol 64:124–127, 1975
9. Remington RD, Schork MA: Statistics With Applications to the Biological and Health Sciences. Prentice-Hall, Englewood Cliffs, N.J., 1970
10. Fleiss JL: Statistical Methods for Rates and Proportions. John Wiley & Sons, New York, 1973.

ANNOUNCEMENT

The Center for Disease Control of the Public Health Service is again offering a continuing series of short, intensive laboratory training courses during 1979-1980. Information and applications may be obtained from the registrar, Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia 30333.

Clin Exp Immunol 1995; 100:126–132

Anti-inflammatory activity of phosphodiesterase (PDE)-IV inhibitors in acute and chronic models of inflammation

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SUMMARY

Inhibitors of cyclic nucleotide phosphodiesterases are known to suppress lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) production *in vitro* in human monocytes. The most potent of these have selectivity for type IV PDEs, suggesting that this class of PDE is the major type involved in the regulation of human TNF-α production. Using compounds of two distinct chemical structural classes, a quinazolinedione (CP-77 059) and a 4 arylpyrrolidinone (rolipram), we show here that PDE-IV-specific inhibitors are also potent in suppressing LPS-induced TNF-α production *in vitro* in sodium periodate-elicited murine macrophages (IC_{50}s of 1 and 33, respectively). We then report the *in vivo* anti-inflammatory effect of PDE-IV inhibition in five murine models of inflammation: (i) elevation of serum TNF-α induced by a sublethal LPS injection; (ii) LPS-induced endotoxic shock; (iii) LPS/galactosamine-induced endotoxic shock; (iv) carrageenan-induced paw oedema; and (v) adjuvant arthritis. Following a sublethal (5 μg/mouse) injection of LPS, serum TNF-α levels in mice peaked sharply, reaching concentrations of 3–12 ng/ml 90 min after injection. In this sublethal LPS assay, CP-77 059 was about 30 times more potent than rolipram, with a minimum effective dose of 0·1 mg/kg *versus* 3 mg/kg for rolipram. This rank order is in keeping with the relative *in vitro* IC_{50}s for CP-77 059 and rolipram, as well as their relative Ki against the human PDE-IV enzyme (46 nM and 220 nM, respectively). In LPS-induced endotoxic shock, rolipram and CP-77 059 at relatively high doses of 30 and 10 mg/kg, respectively, significantly reduced serum TNF-α levels, and also inhibited mortality 66%. In the LPS/galactosamine shock model, in which mice are rendered exquisitely sensitive to LPS by co-injection with galactosamine, only 0·1 μg of LPS/mouse is necessary for serum TNF-α elevation and death. Both rolipram and the CP-77 059 caused dose-dependent reduction of serum TNF-α and lethality. In the carrageenan-induced paw oedema model, in which there is a pronounced local TNF-α response (without a serum TNF-α elevation), rolipram significantly inhibited paw swelling as well as localized TNF-α levels in the paw. In the adjuvant arthritis model, a chronic model of inflammation also possessing localized TNF-α elevation in the inflamed paw, rolipram and CP-77 059 suppressed ankle swelling and radiological evidence of joint damage. These data are consistent with a major role for PDE-IV in regulation of TNF-α production and inflammatory responses in murine systems. It suggests a potential therapeutic use for PDE-IV-specific inhibitors in inflammatory disease such as rheumatoid arthritis, septic shock and other inflammatory diseases where TNF-α has been postulated to be a contributing factor in the pathology of the disease.

Keywords arthritis *in vivo* PDE-IV inhibitors rolipram shock tumour necrosis factor-alpha

INTRODUCTION

The intracellular concentration of cAMP appears to play a major role in the response of inflammatory cells to a wide range of stimuli [1–4]. This is supported primarily by the similar effects on cell function of a variety of cyclic nucleotide-elevating agents including cAMP analogues such as dbcAMP, agents which stimulate adenylate cyclase activity such as prosta-

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glandin E_1 (PGE_1), forskolin and β2-agonists, and cyclic nucleotide phosphodiesterase inhibitors which slow the catabolism of cAMP and cGMP. One of the most striking examples is the suppressive effect of these agents on the production of tumour necrosis factor-alpha (TNF-α) monocytes and macrophages [5–10].

The relatively recent characterization of several PDE isoforms exhibiting differential regulation and tissue expression, and the availability of inhibitors selective for some of these isoforms, has spurred efforts to develop further PDE isoform-selective inhibitors as tools for investigating PDE function and as potential therapeutics. Based on the effect of prototypic PDE-IV selective inhibitors such as rolipram, and molecular cloning efforts, PDE-IV is thought to be a major phosphodiesterase isoform responsible for catabolism of cAMP and regulation of inflammatory function in many cells, including monocytes, lymphocytes, mast cells, basophils and neutrophils [1–4,11].

Like IL-1β, TNF-α is a pluripotent cytokine believed to be involved in the pathology of a variety of inflammatory diseases [12–14]. TNF-α bears many of the activities of IL-1, such as induction of bone resorption, activation of collagenase, stimulation of prostaglandin release and up-regulation of endothelial cells [14]. Again like IL-1β, TNF-α appears to play a key role in septic shock models, as demonstrated by Pfeffer *et al.* [15]. Presence of localized and systemic TNF-α in acute and chronic animal models of inflammation has been shown by Sekut *et al.* [16] and Smith-Oliver *et al.* [17].

In the present study, we investigate the effect of PDE-IV-selective inhibitors, representing two distinct chemical structural classes, on *in vitro* and *in vivo* murine TNF-α production, and on the inflammatory response in several murine inflammation models.

MATERIALS AND METHODS

Animals

Female inbred C57Bl/6, C3H/hen and C3H/HeJ mice (approximately 22 g each) were obtained from Charles River Laboratories Inc. (Raleigh, NC). Rats used in the carrageenan oedema studies were 250-g male, Lewis rats purchased from Charles River Labs. In the adjuvant arthritis model, male Lewis rats 160–170 g from Charles River Labs were free of pathogenic viruses as determined by a standard viral titre screen (Microbiological Associates, Bethesda, MD).

PDE inhibitors

The following PDE inhibitors have been previously described and were synthesized in house for these studies: PDE-IV-specific inhibitors rolipram and CP-77059 [18]; PDE-III-specific inhibitor CI-930; PDE-V inhibitor zaprinast (Verghese *et al.*, J Pharm Exp Ther, accepted for publication, 1995).

TNF-α production by murine macrophages

C3H/HeJ female mice were injected intraperitoneally with 2 ml of 5 mM $NaIO_4$. Five days later, mice were euthanized and peritoneal exudate cells collected into cold PBS. Erythrocytes were lysed with ammonium chloride if necessary. Cells were plated at 5×10^5 cells/well in a 24-well tissue culture plate and allowed to adhere for 1·5 h. Non-adherent cells were removed and test PDE inhibitors were added in RPMI medium with 1% fetal bovine serum (FBS). Lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:B4; Sigma Chemical Co., St Louis, MO; 2·5 ng/ml final concentration) was then added and cultures were incubated for 24 h at 37°C. TNF-α protein was quantified in the supernatant fluids by a commercial murine TNF-α ELISA kit (Genzyme Diagnostics, Cambridge, MA).

Sublethal LPS injection

C3H/hen mice received an i.p. injection of 5 μg LPS in 0·5 ml PBS. Mice were bled from the abdominal vein 90 min following i.p. injection ($n = 4$). The blood was allowed to clot overnight at 4°C and then centrifuged for 15 min. Serum TNF-α levels were measured by ELISA kit.

The PDE-IV inhibitors were suspended in a 0·1% methyl cellulose solution and ground in a homogenizer (Eberbach) to ensure a uniform suspension. A 0·5-ml volume of compound was administered to each mouse by oral gavage 30 min before i.p. injection of LPS. Mice were fasted overnight before dosing.

Endotoxic shock models

Both endotoxic shock models were very similar. They differ mainly in the amount of LPS used and the additional inclusion of galactosamine. In the shock model with LPS alone, 500 μg/mouse of LPS were injected intraperitoneally into C3H/hen mice. In the LPS/galactosamine model of shock, C57/Bl mice received an i.p. injection of 0·5 ml of a mixture of LPS (0·1 μg/mouse) and galactosamine (600 mg/kg; Aldrich Chemical Co., Milwaukee, WI) as previously described [16]. Compounds were administered and serum was obtained and analysed as in the sublethal model ($n = 4$). The survival rate was assessed over a 24-h period ($n = 6$).

Carrageenan-induced paw oedema in rats

A 0·1-ml volume of a 1% solution of carrageenan was injected into the rat hind paw. Animals, previously fasted overnight, were dosed orally with compound in 0·1% methyl cellulose 1 h before carrageenan injection. Three hours after injection, animals were euthanized and paw swelling was measured with calipers. Each group contained eight animals.

For measurement of local TNF-α, paws were removed, weighed, snap-frozen in liquid N_2, pulverized and homogenized for 2 min in 10 ml saline at 4°C as previously described [16]. The suspension was centrifuged in a microcentrifuge to remove debris, and the supernatant assayed using the TNF-α ELISA kit.

Adjuvant arthritis in rats

Rats were injected subcutaneously in the base of the tail with 0·05 ml of Freund's complete adjuvant (FCA) containing 300 μg of *Mycobacterium tuberculosis* (Difco Labs, Detroit, MI) as described previously [19]. Compounds were suspended in a 0·1% solution of methyl cellulose, ground in a homogenizer, and administered by oral gavage once daily in a volume of 2 ml. Ankle diameter was measured throughout the time-course using calipers. At the end of the experiment, rats were euthanized, paws were removed and radiological analysis was performed as previously described [20]. Ankles were scored in a blinded fashion, and each of the five parameters were evaluated on a scale of 0–4. The features given a radiological score were (i) bone demineralization, (ii) bone erosion, (iii) periostitis, (iv) cartilage space reduction, and (v) soft tissue swelling. Thus the

Table 1. *In vitro* effect of phosphodiesterase inhibitors on lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF) production in murine peritoneal macrophages

	Per cent inhibition*			
Concentration (μM)	CP-77 059	Rolipram	CI930	Zaprinast
0·001	55	9	5	0
0·01	67	39	11	8
0·1	77	63	28	5
1	82	77	20	7
10	85	81	49	25

*Represents the mean of at least two experiments.

maximum total score could be 20 [20]. Each group contained eight animals.

Statistical analysis

Comparison of mean serum TNF inhibition and paw swelling was tested between groups using analysis of variance followed by Dunnett's test for multiple comparisons. Lethality rates were compared using Fisher's exact test. Group comparisons where multiple measurements were made on each animal were tested using a multivariate analysis of variance with repeated measures model. Alpha levels for all tests were set at 5%.

RESULTS

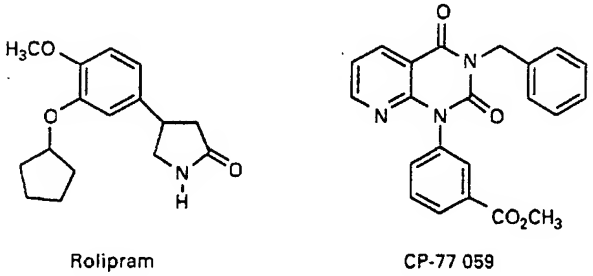
TNF-α production by murine macrophages in vitro

The PDE-IV selective inhibitors rolipram and CP-77 059 were potent suppressors of LPS-induced TNF-α production in Na periodate-elicited mouse peritoneal macrophages, with IC_{50}s of approximately 33 nM and 1 nM, respectively (Table 1). This rank order of potency is the same when the respective Ki against the human PDE-IV enzyme are calculated; with rolipram possessing a Ki of 220 nM ($n = 9$) and CP-77 059 exhibiting a Ki of 46 nM ($n = 4$; Feldman *et al.*, accepted for publication, J Med Chem, 1995).

By contrast, the PDE-III-selective inhibitor CI930 [21] was weakly active (approximately 10 μM), and zaprinast, a PDE-V inhibitor [22,23] had little or no effect (≥ 10 μM).

Sublethal injection of LPS

As an extension of studies of TNF-α inhibition *in vitro*, PDE-IV inhibitors were tested *in vivo*. Mice were injected intraperitoneally with 5 μg LPS, a dose calibrated to elevate serum



Rolipram CP-77 059

Fig. 1. Structure of the PDE-IV inhibitors, rolipram and CP-77 059.

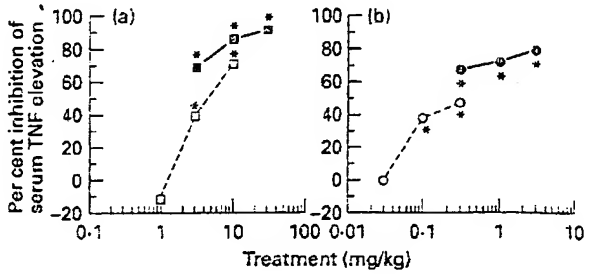


Fig. 2. The effect of PDE-IV inhibitors on serum tumour necrosis factor-alpha (TNF-α) levels in mice injected with a sublethal concentration of lipopolysaccharide (LPS). Fasted female C3H/hen mice were injected intraperitoneally with 5 μg LPS. Rolipram (a) or CP-77 059 (b) was given orally 30 min before LPS injection ($n = 4$). Blood was taken from the heart 90 min after LPS injection and serum TNF-α levels measured using a commercial available ELISA kit. *Significantly different ($P < 0·05$) from LPS control using Dunnett's multiple comparison. ■,●, Test 1; □,○, test 2.

TNF-α levels but not to cause death. Rolipram and CP-77 059 (Fig. 1) were given orally 30 min before LPS injection. In two experiments, rolipram was administered at doses ranging from 1 to 30 mg/kg (Fig. 2a). Rolipram had a minimum effective dose of 3 mg/kg, with maximum inhibition of 92% at 30 mg/kg and no effect at 1 mg/kg. Similar testing of CP-77 059 resulted in a minimum effective dose of 0·1 mg/kg (Fig. 2b). Thus CP-77 059 was about 30-fold more potent than rolipram. Background levels of serum TNF-α in normal mice were undetectable (< 50 pg/ml), while serum TNF-α levels in the LPS-injected mice ranged from 3 to 12 ng/ml.

LPS and LPS/galactosamine-induced endotoxic shock

Since PDE-IV inhibitors were active in their inhibition of low-dose LPS-induced serum TNF-α elevation, these same inhibitors were tested in models of endotoxic shock, thought to depend upon the presence of TNF-α. In the high-dose LPS shock model, serum TNF-α levels were evaluated over time and the 24-h mortality rate recorded (Fig. 3). Just as in the sublethal model, serum TNF-α levels peaked at 90 min post-injection. At high doses (10–30 mg/kg), CP-77 059 and rolipram reduced mortality rate 66% and serum TNF-α level 81–87%.

In the galactosamine (600 mg/kg) + LPS (0·1 μg/ml) model of endotoxic shock, galactosamine destroyed liver function so that normally non-lethal doses of LPS now caused death. In a dose response experiment, rolipram (1, 10 and 30 mg/kg) and CP-77 059 (1, 3 and 10 mg/kg) inhibited serum TNF-α between 84% and 94% (Fig. 4). Rolipram at the highest dose significantly inhibited mortality rate by 66%, while CP-77 059 at doses of 3 and 10 mg/kg also significantly inhibited mortality by 50–66%. However, both drugs failed to significantly block mortality at the lower doses.

Carrageenan oedema

Since elevated TNF-α levels have been observed in extracts of rat and mouse paws injected with carrageenan [16], rolipram was tested for anti-inflammatory activity in this model. At a dose of 3, 10 or 30 mg/kg, rolipram significantly inhibited carrageenan paw oedema by 21%, 43% and 45%, respectively (Fig. 5). It also significantly inhibited TNF-α levels in homogenates from the carrageenan-injected paws, with approxi-

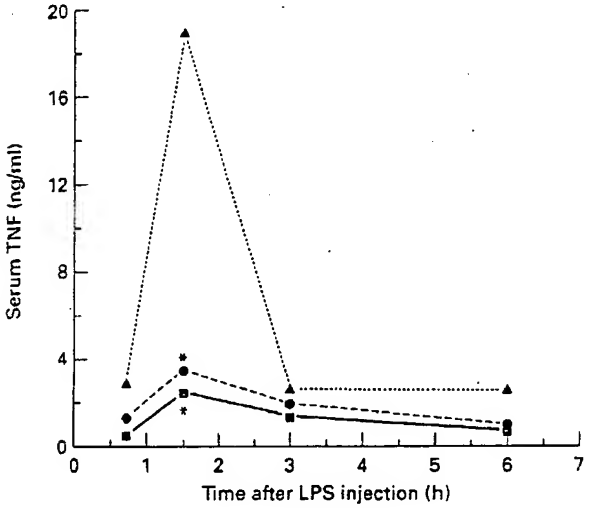


Fig. 3. Effect of PDE-IV inhibitors on serum tumour necrosis factor-alpha (TNF-α) levels and the lethality associated with the lipopolysaccharide (LPS) model of endotoxic shock. Fasted female C3H/hen mice were injected intraperitoneally with 500 μg LPS (n = 22). Four animals were bled at each time point. Six animals were observed for 24 h to establish mortality rate. For further details see Fig. 2 or Materials and Methods. *Significantly different ($P < 0.05$) from LPS control using Dunnett's multiple comparison. ▲, LPS control; ■, rolipram 30 mg/kg; ●, CP-77 059 10 mg/kg.

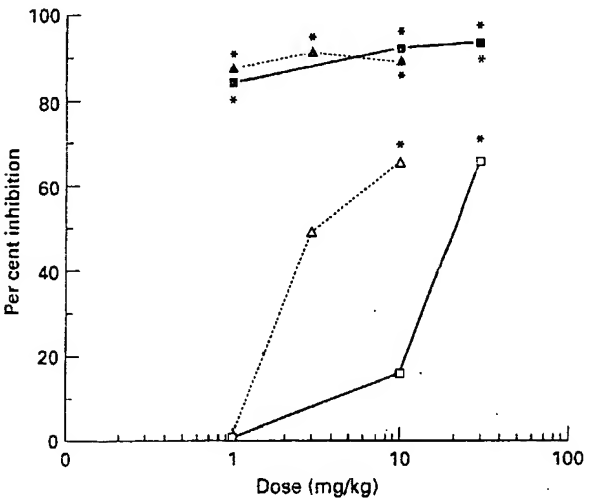


Fig. 4. Effect of PDE-IV inhibitors on serum tumour necrosis factor-alpha (TNF-α) levels and the lethality associated with the lipopolysaccharide (LPS)/galactosamine model of endotoxic shock. Fasted female C57/Bl mice were injected intraperitoneally with 0·1 μg LPS and 600 mg/kg galactosamine (n = 10). Four animals were bled 90 min after LPS injection. Six animals were observed for 24 h to establish mortality rate. For further details see Fig. 2 or Materials and Methods. *Significantly different ($P < 0.05$) from LPS control using Dunnett's multiple comparison. △, CP-77 059 inhibition of lethality; ▲, CP-77 059 inhibition of TNF; □, rolipram, inhibition of lethality; ■, rolipram, inhibition of TNF.

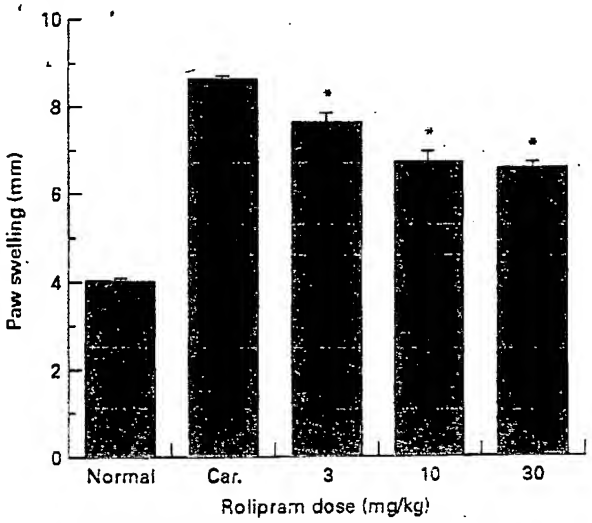


Fig. 5. Effect of rolipram on carrageenan paw oedema. Fasted male Lewis rates were injected in the footpad with 0·1 ml of a 1% solution of carrageenan. Rolipram was given orally 1 h before carrageenan injection (n = 8). Paw diameter was measured with calipers 3 h after carrageenan injection. *Significantly different ($P < 0.05$) from carrageenan control using Dunnett's multiple comparison.

mately 75% inhibition at the high dose (30 mg/kg). There was no detectable TNF-α in the serum of rats injected in the paw with TNF-α, nor was there any TNF-α in normal paw homogenates.

Adjuvant arthritis

Since PDE-IV inhibitors were successful in reducing serum TNF-α levels and soft tissue swelling, these drugs were tested in a chronic model of inflammation where TNF-α is also thought to play a role in soft tissue swelling [16]. In the 3-week adjuvant arthritic rat model, rats were dosed from day 1 with rolipram and CP-77 059 at 0·3, 1 and 3 mg/kg, po (Fig. 6). Both drugs inhibited ankle swelling in a dose response fashion. Rolipram was significantly inhibitory (32%) at 3 mg/kg, while CP-77 059 significantly inhibited ankle swelling by 40% and 42% at 1 and 3 mg/kg, respectively.

Ankles were also evaluated radiologically using five individually graded parameters (0–4). The features assessed were bone demineralization, bone erosion, periostitis, cartilage space reduction and soft tissue swelling as previously described [20]. As in inhibition of ankle swelling, rolipram and CP-77 059 exhibited a significant and dose-dependent effect on each individual parameter. Results depicting the total radiological score show that rolipram at 3 mg/kg significantly reduced radiological changes by 24%, while CP-77 059 at all doses significantly reduced radiological changes by 34–62% (Fig. 7).

In a second experiment, rolipram was dosed in a therapeutic fashion, from day 7 to day 14. Under this regimen, rolipram again exhibited inhibition of ankle swelling and radiological damage of approximately 60% (data not shown).

DISCUSSION

Agents that elevate the concentration of intracellular cAMP can inhibit inflammatory cell activities such as cytokine pro-

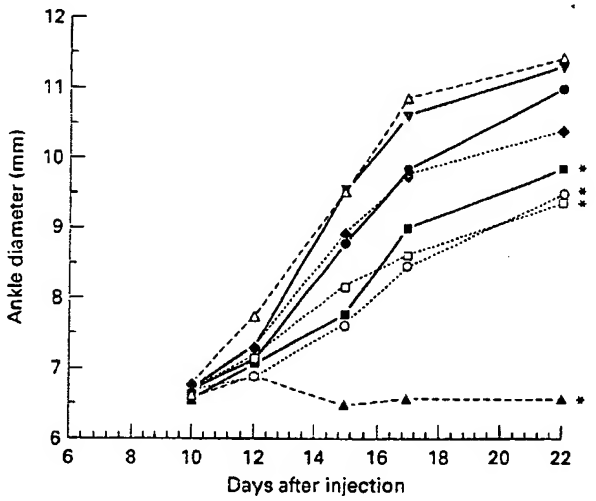


Fig. 6. Inhibition of ankle swelling in adjuvant arthritic rats treated with PDE-IV inhibitors. Male Lewis rats were injected subcutaneously in the tail with 0·05 ml Freund's complete adjuvant. Animals were dosed orally from day 1 to day 21 with rolipram or CP-77 059 ($n = 8$). Measurement of ankle swelling was taken throughout the time course using calipers. *Significantly different ($P < 0·05$) from arthritic control using Dunnett's multiple comparison. ▲, Normal; △, arthritic; ■, rolipram (3 mg/kg); ●, rolipram (1 mg/kg); ▼, rolipram (0·3 mg/kg); □, CP-77 059 (3 mg/kg); ○, CP-77 059 (1 mg/kg); ◆, CP-77 059 (0·3 mg/kg).

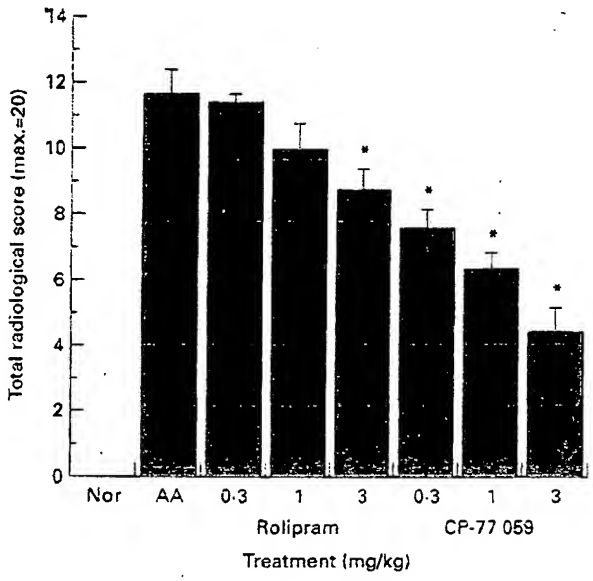


Fig. 7. Radiological score of adjuvant arthritic rats treated with PDE-IV inhibitors. Total radiological score was based on five parameters each scored from 0 (normal) to 4 (severe changes) as previously described [15]. Thus a maximum total score could be 20. For further details see Fig. 6 or Materials and Methods. *Significantly different ($P < 0·05$) from arthritic control using Dunnett's multiple comparison.

duction, chemotaxis, cytotoxicity and cell aggregation [1]. PDE type-IV inhibitors, by virtue of their ability to block phosphodiesterase activity in monocytic cells, thus elevating cAMP levels, may be a class of compounds from which useful anti-inflammatory drugs can be developed [1–4].

This study demonstrated the effectiveness of PDE type-IV inhibitors in various acute and chronic models of inflammation in which TNF-α may have a pathological role. PDE-IV inhibitors were potent suppressors of LPS-induced TNF-α production in murine macrophages, whereas inhibitors selective for PDE-III or -V were much less active. Similar findings have been reported for TNF-α production in human monocytes [5,7], supporting a major role for PDE-IV in the regulation of both human and murine TNF-α production.

The *in vivo* potency of PDE-IV inhibitors was assessed in a mouse model, where LPS was injected at a sublethal concentration to induce appearance of serum TNF-α. The PDE-IV inhibitors rolipram and CP-77 059 were orally active, dose-dependent inhibitors of serum TNF-α levels in this system. These PDE-IV inhibitors were tested in endotoxic shock models, where serum TNF-α levels as well as mortality rate could be measured. In the LPS/galactosamine model of endotoxic shock, rolipram and CP-77 059 not only effectively reduced serum TNF-α levels, but also showed protective effects in a dose-dependent fashion. However, reduction of serum TNF-α level did not guarantee complete protection against lethality, since animals suffered 100% mortality at doses which inhibited serum TNF-α levels over 80%. Since shock is accompanied by a potentially lethal fall in blood pressure, we considered the possibility that the hypotensive properties of the PDE-IV inhibitors were responsible for the difference in the relatively high protective dose *versus* the much lower TNF inhibitory dose. However, we have observed that non-hypotensive compounds such as glucocorticoids also manifested this discrepancy between the dose needed to protect against lethal shock and the dose needed to inhibit serum TNF levels. In addition, the PDE-IV inhibitors are active at high doses, where the hypotensive side-effects are most pronounced. If their haemodynamic profile contributed to their poor potency in the LPS lethality assay, then one would have predicted that the compounds would be active at the low dose (where TNF inhibition was high and hypotensive effect low) and inactive at the high dose (where the TNF inhibition would be countered by the pronounced hypotensive effect). While the haemodynamic effects of PDE-IV inhibitors may contribute to this lack of correlation between survival rate and reduction of serum TNF-α level, it is also possible that the difference may be due to the greater relevance of localized elevation of TNF-α in target tissues such as the lung, liver and paw as opposed to systemic levels in the blood [16]. Alternatively it may also be due in part to the pro-inflammatory activity of cytokines not affected by PDE-IV inhibitors.

Since we have previously demonstrated that elevation of TNF-α occurred in the paw but not in the serum of rats injected with carrageenan [16], we tested rolipram for its anti-inflammatory activity in this model of localized TNF-α elevation. Rolipram and CP-77 059 have previously been shown to suppress carrageenan paw oedema [18]. We have confirmed and extended that observation here by showing that rolipram significantly inhibited paw inflammation as well as local TNF-α production. It cannot be ruled out that the anti-oedema action of these agents may in part be the result of their induction of systemic hypotension. However, because of their profound

effect on neutrophils *in vitro* [21,22], it is likely that a substantial component of the anti-inflammatory activity seen in the carrageenan oedema model is due to inhibition of neutrophil activity *in vivo*.

Encouraged by the activity of these prototypic PDE type-IV inhibitors in models of acute inflammation, we next evaluated the compounds in a chronic model of inflammation. The rat adjuvant arthritis model mimics many aspects of human rheumatoid arthritis, including elevated levels of TNF-α in the arthritic joint [17,24]. In this model, rolipram and CP-77059 not only reduced tissue oedema but also inhibited radiological changes in the bone. In a previous paper we compared ankle swelling and joint TNF-α levels in arthritic rats treated with non-steroidal anti-inflammatory drugs or immunosuppressive compounds [17]. It will also be important to address the relationship between joint protection and joint TNF-α levels in arthritic rats treated with PDE-IV inhibitors.

The most suitable candidates of PDE inhibitors for evaluation in the clinic should possess the following properties: high specificity to a selected PDE-IV isozyme, high potency, low toxicity and good oral absorption. The recognition of multiple isoforms [11,25] and a non-random tissue distribution of PDE-IV [1–4,26] suggest the potential to develop more specific and less toxic PDE-IV inhibitors. Synergism of PDE inhibitors and β2-agonists should be evaluated. In the sublethal LPS model of serum TNF-α measurement, we have observed some mild synergistic effect between salmeterol and rolipram (unpublished data).

In conclusion, we have shown that PDE-IV inhibitors, in addition to being potent suppressors of TNF-α production, are also good anti-inflammatory agents in several models of acute and chronic inflammation. It is conceivable that the reported activities of these compounds are due to unknown reactivities, but the finding that compounds of two different chemical structural classes have similar activities supports the notion that PDE-IV inhibition is relevant. It should be emphasized that while the suppressive effect on TNF-α production is one of the most potent, consistent and complete inhibitory effects known for PDE inhibitors in relation to inflammatory cell function, the extent to which this contributes to anti-inflammatory activity in each of these models will not be clear until the role of TNF-α *in vivo* is better defined. For example, PDE-IV inhibition has been shown to have suppressive effects on several other potentially pro-inflammatory cellular activities, including IL-1β [7] and LTB4 [27] production by monocytes, neutrophil respiratory burst [28], histamine and LTB4 release from basophils [29], histamine and LTC4 from mast cells [27] and T lymphocyte blastogenesis [30]. In addition, there are several examples of cAMP-regulated inflammatory cell function which have not been fully explored with respect to PDE inhibition. Nevertheless, these data suggest that a specific PDE-IV inhibitor has potential therapeutic utility for acute and chronic inflammatory disease, and are consistent with the hypothesis that TNF-α is an important mediator in inflammatory disease.

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REFERENCES

1 Torphy TJ, Undem BJ. Phosphodiesterase inhibitors: new opportunities for the treatment of asthma. Chest 1991; **46**:512–23.
2 Giembycz MA, Dent G. Prospects for selective cyclic nucleotide phosphodiesterase inhibitors in the treatment of bronchial asthma. Clin Exp Allergy 1992; **22**:337–44.
3 Torphy TJ, Livi GP, Christensen SB. Novel phosphodiesterase inhibitors for the therapy of asthma. Drug News and Perspectives 1993; 6:203–14.
4 Lowe III JA, Cheng JB. The PDE IV family of calcium-independent phosphodiesterase enzymes. Drugs of the Future 1992; 17:799–807.
5 Cohan VL, Naclerio BA. LPS-induced tumor necrosis factor alpha (TNFα) release by isolated human monocytes is sensitive to agents that elevate intracellular levels of cyclic AMP. J Immunol 1993; **150** (No 8,Pt 2):212A.
6 Sever A, Rapson NT, Hunter C A, Liew FY. Regulation of tumor necrosis factor production by adrenaline and β-adrenergic agonists. J Immunol 1992; **148**:3441–5.
7 Molnar-Kimber KL, Yonno L, Heaslip RJ *et al.* Differential regulation of TNF-α and IL-1β production from endotoxin stimulated human mocytes by phosphodiesterase inhibitors. Mediator Infl 1992; 1:411–7.
8 Semmler J, Wachtel H, Endres S. The specific type IV phosphodiesterase inhibitor rolipram suppresses tumor necrosis factor-production by human mononuclear cells. Int J Immunopharmacol 1993; 15:409–13.
9 Renz H, Gong JH, Schmidt A *et al.* Release of tumor necrosis factor from macrophages: enhancement and suppression are dose dependently regulated by prostaglandin E_2 and cyclic nucleotides. J Immunol 1988; **144**:2388–93.
10 Sekut L, Champion BR, Page K, Menius JA, Connolly K. Anti-inflammatory activity of salmeterol: down regulation of cytokine production. Clin Exp Immunol 1995; **99**:461–6.
11 Livi GP, Kmetz P, McHale MM *et al.* Cloning and expression of cDNA for a human low-K_m, rolipram-sensitive cyclic AMP phosphodiesterase. Mol Cell Biol 1990; **10**:2678–786.
12 Jäättelä M. Biology of disease; biologic activites and mechanisms of action of tumor necrosis factor-α/cachectin. Lab Invest 1991; **64**:724–42.
13 Piquet PF, Grau GE, Vassalli P. Tumor necrosis factor and imunopathology. Immunol Res 1991; 10:122–40.
14 Fiers W. Tumor necrosis factor; characterization at the molecular, cellular and *in vivo* level. FEBS 1991; **285**:199–212.
15 Pfeffer K, Matsuyama T, Kunding TM *et al.* Mice deficient for the 55 kD tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to *L. monocytogenes* infection. Cell (USA) 1993; **73**:457–67.
16 Sekut L, Menius JA Jr, Brackeen MF *et al.* Evaluation of the significance of elevated levels of systemic and localized TNF in different animal models of inflammation. J Lab Clin Med 1994; **124**:S13–20.
17 Smith-Oliver T, Noel LS, Stimpson SA *et al.* Elevated levels of TNF in the joints of adjuvant arthritic rats. Cytokine 1993; **5**:298–305.
18 Lowe JA, Archer RL, Chapin DS *et al.* Structure–activity relationship of quinazolinedione inhibitors of calcium-independent phophodiesterase. J Med Chem 1991; **34**:624–8.
19 Connolly KM, Stecher VJ, Kent L. Examination of interleukin-1 activity, the acute phase response, and leukocyte subpopulactions in rats with adjuvant-induced arthritis. J Lab Clin Med 1988; 111:341–7.
20 Clark RL, Cuttino JT, Anderle SK *et al.* Radiologic analysis of arthritis in rats after systemic injection of streptococcal cell walls. Arthritis Rheum 1979; **22**:25–35.
21 Verghese MW, Brown TA, Irsula O *et al.* Anti-neutrophil activity of cyclic nucleotides with varying cardiotonic potencies. J Mol Cell Cardiol 1989; **21**:S61.

22 Nielson CP, Vestal RE, Sturm RJ *et al.* Effects of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte respiratory burst. J Allergy Clin Immunol 1990; 86: 801–8.
23 Peachell PT, Undem BJ, Schleimer RP *et al.* Preliminary identification and role of phosphodiesterase isoenzymes in human basophils. Immunology 1992; 148:2503–10.
24 Lewis AJ, Carlson RP, Chang J. Experimental models of inflammation. In: Bonta IL, Brag MA, Parnham MJ, eds. The pharmacology of inflammation. New York: Elsevier 1985: 371–97.
25 McLaughlin MM, Cieslinski LB, Burman M *et al.* A low K_m, rolipram-sensitive, cAMP-specific phosphodiesterase from human brain. J Biol Chem 1993; 268:6470–6.
26 Beavo JA, Reifsnyder DH. Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Trends Pharm Sci 1990; 11:150–5.
27 Griswold DE, Webb EF, Breton J *et al.* Effect of selective phosphodiesterase type IV inhibitor, rolipram, on fluid and cellular phases of inflammatory response. Inflammation 1993; 17:333–44.
28 Neilson CP, Vestal RE, Sturm RJ *et al.* Effects of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte respiratory burst. J Allergy Clin Immunol 1990; 86:801–8.
29 Peachell PT, Undem BJ, Schleimer RP *et al.* Preliminary identification and role of phosphodiesterase isozymes in human basophils. J Immunol 1992; 148:2503–10.
30 Robicsek SA, Blanchard DK, Djeu JY *et al.* Multiple high-affinity cAMP-phosphodiesterases in human T-lymphocytes. Biochem Pharmacol 1991; 42:869–77.

11 Woll, P. J. and Rozengurt, E. (1988) *Proc. Natl Acad. Sci. USA* 85, 1859–1863
12 Rozengurt, E. (1988) *Ann. NY Acad. Sci.* 547, 277–292
13 Mahmoud, S., Palaszynski, E., Fiskum, G., Coy, D. H. and Moody, T. W. (1989) *Life Sci.* 44, 367–373
14 Jensen, R. T., Jones, S. W., Folkers, K. and Gardner, J. D. (1984) *Nature* 309, 61–63
15 Coy, D. H. *et al.* (1988) *J. Biol. Chem.* 263, 5056–5060
16 Coy, D. H. *et al.* (1989) *J. Biol. Chem.* 264, 14691–14697
17 Heinz-Erian, P. *et al.* (1987) *Am. J. Physiol.* 252, G439–G442
18 Wang, L-H. *et al.* (1990) *Biochemistry* 29, 616–622
19 Wang, L-H. *et al.* (1990) *J. Biol. Chem.* 265, 15695–15703
20 Gamble, R. *et al.* (1989) *Life Sci.* 45, 1521–1527
21 Heimbrook, D. C. *et al.* (1989) *J. Biol. Chem.* 264, 11258–11262
22 Saari, W. S., Heimbrook, D. C., Friedman, A., Fischer, T. W. and Oliff, A. (1989) *Biochem. Biophys. Res. Commun.* 165, 114–117
23 Jensen, R. T. *et al.* (1988) *Am. J. Physiol.* 254, G883–G890
24 Saeed, Z. A. *et al.* (1989) *Peptides* 10, 597–603
25 Shirakawa, T. and Hirschowitz, B. I. (1985) *Am. J. Physiol.* 249, G668–G673
26 Yachnis, A. T., Crawley, J. N., Jensen, R. T., McGrane, M. M. and Moody, T. W. (1984) *Life Sci.* 35, 1963–1969
27 Pappas, T., Hamel, D., Debas, H., Walsh, J. and Tache, Y. (1984) *Peptides* 6, 1001–1003
28 Broccardo, M., Erspamer, G. F., Melchiorri, P., Negri, L. and De Castiglione, R. (1976) *Br. J. Pharmacol.* 55, 221–224
29 Martinez, J. *et al.* (1985) *J. Med. Chem.* 28, 1874–1879
30 Rossowski, W. J. *et al.* (1989) *Scand. J. Gastroenterol.* 24, 121–128
31 Trepel, J. B. *et al.* (1988) *Biochem. Biophys. Res. Commun.* 156, 1383–1389
32 Holst, J. J., Harling, H., Messell, T. and Coy, D. H. (1990) *Scand. J. Gastroenterol.* 25, 89–96
33 Rivier, J. E. and Brown, M. R. (1988) *Biochemistry* 17, 1766–1771
34 Erne, D. and Schwyzer, R. (1987) *Biochemistry* 26, 6316–6319
35 Heimbrook, D. C. *et al.* (1988) *J. Biol. Chem.* 263, 7016–7019
36 Coy, D. H., Wang, L-H., Jiang, N-Y. and Jensen, R. T. *Eur. J. Pharmacol.* (in press)
37 Spanarkel, M., Martinez, J., Briet, C., Jensen, R. T. and Gardner, J. D. (1983) *J. Biol. Chem.* 258, 6476–6479
38 Coy, D. H. *et al.* (1990) in *Neuropeptides and their Receptors Symposium 29*, (Schwartz, T. W., Hilsted, L. M. and Rehfeld, J. F., eds), *Alfred Benzon* pp. 376–385, Munksgaard
39 Dickinson, K. E. J. *et al.* (1988) *Biochem. Biophys. Res. Commun.* 157, 1154–1158
40 von Schrenck, T. *et al.* (1990) *Am. J. Physiol.* 259, G468–G473
41 Severi, C., Jensen, R. T., Torsoli, A. and Delle Fave, G. (1990) *Gastroenterology* 98, A523
42 von Schrenck, T. *et al.* (1989) *Am. J. Physiol.* 256, G747–G758
43 Ladenheim, E. E., Jensen, R. T., Mantey, S. A., McHugh, P. R. and Moran, T. H. *Brain Res.* (in press)
44 Regoli, D. *et al.* (1988) *Ann. NY Acad. Sci.* 547, 158–173
45 Falconieri Erspamer, G. *et al.* (1988) *Regul. Pept.* 21, 1–11
46 Lee, M. C., Jensen, R. T., Coy, D. H. and Moody, T. W. *J. Mol. Cell. Neurosci.* (in press)
47 Westendorf, J. M. and Schonbrunn, A. (1983) *J. Biol. Chem.* 258, 7527–7535

Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes

C. David Nicholson, R. A. John Challiss and Mohammed Shahid

Since the discovery of cyclic nucleotide phosphodiesterase 30 years ago, there have been major advances in our knowledge of this group of isoenzymes. Five families, each composed of several isoforms and having differing tissue distributions, have been described. David Nicholson and colleagues *compare the tissue distribution of phosphodiesterase isoenzymes and discuss the differential effects of inhibition of particular isoenzymes, with differing subcellular localization, on tissue function. They also review the potential use of isoenzyme selective phosphodiesterase inhibitors in a range of clinical disorders such as heart failure, asthma, depression and dementia.*

Cyclic nucleotide phosphodiesterases (EC 3.1.4.17) play a key role in the metabolism of cAMP and cGMP. Many agents modulate tissue function via stimulation of adenylyl or guanylyl cyclase activity and hence via the elevation of cellular levels of cAMP and cGMP. Phosphodiesterase activity moderates the effect of these agents by increasing the rate of breakdown of these cyclic nucleotides. The existence of families of phosphodiesterase isoenzymes is well recognized and isoenzyme selective inhibitors have been identified[1]. A recent *TiPS* review[2] summarized our knowledge of the structure, regulation and some aspects of the localization of phosphodiesterase isoenzymes; it also proposed a nomenclature for the identified isoenzyme families. This review further compares the tissue distribution of phosphodiesterase isoenzymes, describes how selective inhibitors can differentially modulate both cellular cyclic nucleotide levels and tissue function and discusses the clinical potential of phosphodiesterase isoenzyme selective inhibitors.

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Comparative tissue distribution

The nomenclature used for the phosphodiesterase isoenzymes, their most salient regulatory characteristics and substrate specificities, as well as the pharmacological profile of representative isoenzyme selective inhibitors, is summarized in Table I. A chromatographic elution profile illustrating the phosphodiesterase isoenzymes in cardiac muscle is shown in Fig. 1. Although early studies were performed with low-resolution techniques, sufficient data are now available to illustrate clear tissue differences in phosphodiesterase isoenzyme distribution. For example, PDE I, PDE III and PDE IV represent more than 90% of the total cAMP phosphodiesterase activity in brain, platelet and kidney cells, respectively[1,2]. Table II summarizes results from recent studies that included appro-

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TABLE I. Cyclic nucleotide phosphodiesterase isoenzymes

Isoenzyme family*	Regulatory characteristics*	Alternative classification†	Isoenzyme selective inhibitors‡	Additional activities	Functional effects of inhibitors
I	Dependent upon Ca^{2+} and calmodulin, isoforms isolated both within and between tissues with differing K_m values for cAMP and cGMP[a–f]	I[e,k–o] PI[b] Ic[c], PIIp, F2[h,i]	vinpocetine[h,j]	adenosine uptake inhibition[bb]	modulation of central function[ii]; vascular smooth muscle relaxation[h,j]
II	The cAMP hydrolytic activity is stimulated by cGMP; high K_m for cAMP and cGMP[b,f]	II[c,e,k,l,o,q] PII[b]	selective inhibitors not reported		unknown
III	Hydrolytic activity for cAMP inhibited by cGMP; low K_m for cAMP and for cGMP[b,c,e–g]	III[c,e,k–n], IV[o], F3[h,j], PIV[b], PIII[p]	milrinone[l,m,q] piroximone[k] pimobendan[m] cilostamide[r] SK&F94120[o,p] RS82856[l] imazodan[k,q] Org30029[t] ICI118233[u] zardaverine[v] EMD54622[w] indolidan[x] enoximone[x]	adenosine antagonism[cc] adenosine antagonism[cc] Ca^{2+} sensitization[dd] Ca^{2+} sensitization[ee]	positive inotropism[m], vascular[y] and airways smooth muscle relaxation[c], platelet aggregation inhibition[p], stimulation of lipolysis[r]
IV	No known regulator; low K_m only for cAMP[b,c,e–g]	IV[c,e,o], PIII[b]	rolipram[e] RO20–1724[c,e,t] EMD54622[w] denbufylline[b] etazolate[y] Org30029[t] ICI63197[z] zardaverine[v]	high-affinity site[ff] adenosine antagonism[b] adenosine antagonism[gg] Ca^{2+} sensitization[ee]	airway smooth muscle relaxation[c], inhibition of inflammatory mediator release[jj], modulation of central function[kk], gastric acid secretion[ll]
V	Considerable evidence for different isoforms with high and low K_m values for cGMP; evidence for G protein control of light-sensitive isoform[f,h–j]	V, Ia[c], III[o], PI[p], F1[h,j]	MY5445[h] zaprinast[aa] dipyridamole[l,aa]	adenosine uptake inhibition[hh]	platelet aggregation inhibition[h]

*Isoenzymes are grouped in families as suggested in Ref. 2 (main reference list).

†Cyclic nucleotide isoenzymes have been classified according to a number of different schemes, many based on the order of elution from chromatography columns. Because tissue isoenzyme distribution and separation conditions vary, the order of elution varies between laboratories. The table compares the nomenclature used for isoenzymes with the same regulatory characteristics between laboratories. P, peak; F, fraction.

‡Nonselective inhibitors include the alkylxanthines 2-isobutyl-1-methyl xanthine (IBMX), theophylline, caffeine, and pentoxifylline and papaverine.

[a]Kincaid, R. L. *et al.* (1984) *J. Biol. Chem.* 259, 5158–5166; [b]Nicholson, C. D. *et al.* (1989) *Br. J. Pharmacol.* 97, 889–897; [c]Torphy, T. J. (1988) in *Directions for New Anti-Asthma Drugs* (O'Donnell, S. R. and Persson, C. G. A., eds), pp. 37–58, Birkhauser-Verlag; [d]Sharma, R. K. and Wang, J. H. (1985) *Proc. Natl Acad. Sci. USA* 82, 2603–2607; [e]Reeves, M. L. *et al.* (1987) *Biochem. J.* 241, 535–541; [f]Beavo, J. (1988) in *Advances in Second Messenger and Phosphoprotein Research* (Vol. 22) (Greengard, P. and Robison, G. A., eds), pp. 1–38, Raven Press; [g]Yamamoto, T. *et al.* (1984) *Biochemistry* 23, 670–675; [h]Hidaka, H. and Endo, T. (1984) in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Vol. 16) (Strada, S. J. and Thompson, W. J., eds), pp. 245–259, Raven Press; [i]Ahn, H. S. *et al.* (1989) *Biochem. Pharmacol.* 38, 3331–3339; [j]Stryer, L. *et al.* (1981) in *Current Topics in Membranes and Transport* (Miller, H., ed.), pp. 93–108, Academic Press; [k]Weishaar, R. E. *et al.* (1985) *J. Med. Chem.* 28, 537–545; [l]Silver, P. J. *et al.* (1988) *Eur. J. Pharmacol.* 140, 85–94; [m]Brunkhorst, D. *et al.* (1989) *Naunyn Schmiedeberg's Arch. Pharmacol.* 339, 575–583; [n]Komas, N. *et al.* (1989) *J. Cardiovasc. Pharmacol.* 14, 213–220; [o]Appleman, M. M. *et al.* (1984) in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Vol. 16) (Strada, S. J. and Thompson, W. J., eds), p. vi, Raven Press; [p]Simpson, A. W. M. *et al.* (1988) *Biochem. Pharmacol.* 37, 2315–2320; [q]Weishaar, R. E. *et al.* (1984) *Biochem. Pharmacol.* 35, 787–800; [r]Elks, M. L. and Manganiello, V. C. (1984) *Endocrinology* 15, 1262–1268; [s]Alvarez, R. *et al.* (1986) *Mol. Pharmacol.* 29, 554–560; [t]Shahid, M. *et al.* (1989) *Br. J. Pharmacol.* 96, 186P; [u]Pyne, N. J. *et al.* (1987) *Biochem. J.* 24, 897–901; [v]Galvan, M. *et al.* (1990) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 342, 221–227; [w]Klockow, M. *et al.* (1989) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 339, R53; [x]Kauffman, R. F. *et al.* (1987) *J. Pharmacol. Exp. Ther.* 242, 864–872; [y]Chasin, M. *et al.* (1972) *Biochem. Pharmacol.* 21, 2443–2450; [z]Pyne, N. J. *et al.* (1987) *Biochem. J.* 242, 33–42; [aa]Lugnier, C. *et al.* (1986) *Biochem. Pharmacol.* 35, 1743–1751; [bb]Fredholm, B. B. *et al.* (1983) *Acta Pharmacol. Toxicol.* 52, 236–244; [cc]Parsons, W. J. *et al.* (1988) *Mol. Pharmacol.* 33, 441–448; [dd]van-Meel, J. C. (1987) *Arzneim.-Forsch. Drug Res.* 37, 679–687; [ee]Miller, D. J. and Steele, D. S. (1990) *Br. J. Pharmacol.* 100, 843–849; [ff]Schnieder, H. H. *et al.* (1986) *Eur. J. Pharmacol.* 127, 105–115; [gg]Williams, M. *et al.* (1988) *Pharmacol. Biochem. Behav.* 29, 434–441; [hh]Marangos, P. J. *et al.* (1985) *Eur. J. Pharmacol.* 117, 393–394; [ii]de Noble, V. *et al.* (1986) *Pharmacol. Biochem. Behav.* 24, 1123–1128; [jj]Verghese, M. W. *et al.* (1989) *J. Mol. Cell. Cardiol.* 12 (Suppl. II), S.61; [kk]Wachtel, H. (1983) *Neuropharmacology* 27, 267–272; [ll]Black, E. W. *et al.* (1988) *J. Pharmacol. Methods* 20, 57–58.

priate tests to distinguish phosphodiesterase isoenzymes (see Table II for Refs). Other tissues of note, not listed in Table II, are retina and adrenal cortex whose cells contain almost exclusively PDE V and PDE II, respectively. Preliminary studies have also shown that a variety of inflammatory cells contain high levels of PDE IV activity, but current information on other phosphodiesterase isoenzymes in these cells is lacking.

There are also tissue-specific differences in isoforms within one phosphodiesterase isoenzyme family. This point is illustrated by the different substrate specificities of PDE I found in liver, brain and fat cells (high affinity for cGMP, low affinity for cAMP) when compared to that found in the heart and kidney (high affinity for both cAMP and cGMP). Both brain and testicular tissue contain multiple forms of PDE I, while liver, kidney,

skeletal muscle and airways smooth muscle cells all contain more than one PDE IV isoform. The PDE IV subtypes found in both liver and kidney show 5–15-fold differences in their sensitivity to the selective inhibitors rolipram and Ro20-1724 (Refs 3, 4). Whether these subtypes are distinct isoenzymes, differently charged isomers or proteolytic by-products of one enzyme awaits clarification.

A novel IBMX-insensitive and Mg^{2+}-independent, cAMP-specific phosphodiesterase in liver cells[3], which does not easily fit into the present classification, has also been described. A major difficulty in analysing tissue differences in phosphodiesterase isoenzyme distributions is the fact that major species differences are also apparent; further analysis of phosphodiesterase isoenzyme profiles in human tissue is clearly required to aid drug development.

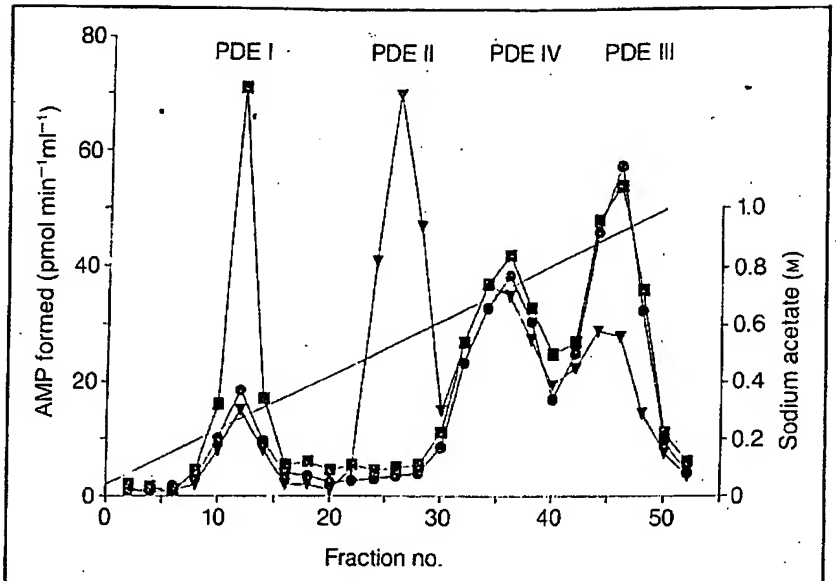

Fig. 1. Typical separation profile of cyclic nucleotide phosphodiesterase (PDE) activities from rat cardiac ventricle. Supernatant from cardiac homogenates was applied to Q-Sepharose Fast Flow columns and activities eluted with a linear gradient of sodium acetate in buffer. The eluate was collected in 7 ml fractions and assayed for cAMP hydrolytic activity. Fractions were assayed with 1 μM cAMP (•), 1 μM cAMP in the presence of Ca^{2+}/calmodulin (■) and 1 μM cAMP in the presence of cGMP (▼). Four distinct PDE isoenzymes are clearly distinguishable. (From Ref. 31.)

Subcellular distribution of phosphodiesterase isoenzymes

It is likely that the intracellular distribution of phosphodiesterase isoenzymes is an important factor in determining their regulatory role. In the majority of early studies examining phosphodiesterase isoenzyme subcellular distribution, isoenzymes were separated from the supernatant fraction of homogenized tissue using techniques (e.g. hypotonic buffers, lack of protease inhibitors, sonication, presence of detergents, vigorous homogenization) that may have displaced membrane-bound phosphodiesterase; hence both cytosolic and particulate isoenzymes may have been measured. More recent experiments have used more appropriate extraction methods (see Table II). In general, there are very marked differences in the relative phosphodiesterase activity levels of particulate and cytosolic fractions in different tissues; cardiac, liver and fat cells contain high (50–70%) levels of membrane-bound phosphodiesterase activity, whereas in smooth muscle, kidney and platelet cells, most (80–90%) of the phosphodiesterase activity is present in the cytosolic fraction.

A variety of organelles possess phosphodiesterase activity; however, the functional relevance of the occurrence of phosphodiesterase activity in some organelles (e.g. mitochondria, nucleus, myofibrils) remains obscure.

Regulation of cyclic nucleotide levels

Characterization of tissue-specific phosphodiesterase isoenzymes is insufficient to predict the effects of selective phosphodiesterase inhibition in intact tissue preparations. A crucial step in such investigations is the determination of the relative importance of the phosphodiesterase isoenzymes for cyclic nucleotide hydrolysis; as yet, however, there is still relatively little definitive information.

The first experiments to explore the role of phosphodiesterase isoenzymes in the regulation of cyclic nucleotide hydrolytic rates in intact tissues were performed in vascular smooth muscles[5,6]. In these studies, the ability of various phosphodiesterase inhibitors to potentiate sodium nitroprusside- and isoprenaline-induced relaxation of vascular tissue was investigated, and the importance of PDE I and PDE V in cGMP hydrolysis, and of PDE III in cAMP hydrolysis, was established.

More studies have focused on the role of PDE III in the regulation of cAMP hydrolysis in airways[7,8], vascular smooth muscle[9] and myocardial tissue[10–12]. In vascular smooth muscle there is an excellent correlation between relaxation of 5-HT-induced contractions and elevation of cyclic nucleotides[9]. In cardiac muscle, the demonstration of correlation between the cAMP elevation induced by PDE III inhibition and inotropic responses has been equivocal[11] (this is the case for inotropy mediated either via β-adrenoceptor stimulation or inhibition of cyclic nucleotide hydrolysis). It is now generally accepted that PDE III inhibitors do produce positive inotropy through cAMP-dependent mechanisms[11] and this apparent discrepancy may be explained in terms of compartmentation of cAMP and/or cAMP-dependent protein kinase. Microcompartmentation of the components of the cAMP signal transduction pathway is well established. cAMP-elevating agents vary in their ability to activate cAMP-dependent protein kinase in perfused heart preparations[13], and it is possible that elevation of cAMP (e.g. via phosphodiesterase inhibition) in a particular cellular compartment is necessary for the induction of this response. Detailed studies in cerebral cortex[14] slices have shown that selective inhibitors of PDE IV cause elevation of cAMP concentration and poten-

TABLE II. Tissue distribution and intracellular localization of cyclic nucleotide phosphodiesterase isoenzymes

	Soluble PDE				Particulate PDE				
Tissue	Isoenzyme	Species	Methods*	% Activity	Isoenzyme	Species	Methods*	Organelle†	% Activity
Cardiac Ventricle	I, II, III, IV	human[a]	A	23–40[b]	III	human[c]	D‡	–	60–70[b]
		rabbit[d,e]	A	47–60[f,g]	III	rabbit[f,g]	A	SR (~1%)	39–50[f,g]
		guinea-pig[a]	A	–	IV	guinea-pig[h]	A	myofibrils	–
		dog[i,j]	A	–	III	dog[i,j]	A	mito/SR	15[j,k]
		rat[l]	C	51–69[m]	III/IV	dog[n]	A	–	–
	I, II, III	human[c]	D	–	IV	rat[l]	A	–	40–50[l]
	I, III, IV	dog[o]	D	–	–	rat[m]	B	nucleus (34%) mito (9%) SL/SR (6%)	55–65[m]
					II	rat[p]	D	–	3[p]
					II	frog[q]	A	–	50[q]
S-A node	I, II, III, IV	dog[n]	A	–	III/IV	dog[n]	A‡	–	–
Liver	I, II, III/IV§	human[b]	A	60[b]	–	human[b]	A	–	35–42[b]
	I, II, IV	rat[p]	D	45–50[r]	III	rat[s]	D	dense vesicle	–
	I, II, III, IV	calf[t]	C	72–82[m]	IV	rat[s]	D	PM	–
					II	rat[u]	D	PM	–
					–	rat[m]	B	nucleus (7%) mito (14%) microsomes (7%)	21[m]
Brain	I, II, III/IV§	human[b]	A	30–50[b]	IV	rat[v]	D	–	–
	I, II, IV	human[w]	C	–	II	rabbit[x]	D	–	75[x]
					–	pig[y]	D	nucleus (24%) mito (32%) microsomes (28%)	64[y]
	I, II, IV	rat[l]	C‡	–					
Aorta	I, III/IV§, V	rabbit[z]	A	–	III	guinea-pig[aa]	B	SL	–
	I, IV, V	bovine, human and rat[bb]	A	–					
	I, III/IV§, V	bovine[cc]	C	–					
	I, III/IV§, V	pig[dd]	A	80–90[dd]					

*A, Hypotonic buffer, polytron homogenization; B, isotonic buffer, polytron homogenization; C, hypotonic buffer, Potter–Elvehjem/Dounce homogenization; D, isotonic buffer, Potter–Elvehjem/Dounce homogenization.

†SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; SL, sarcolemma; PM, plasma membrane; mito, mitochondria.

‡Detergent solubilized, other particulate characterizations solely based on regulatory characteristics of a possible mixture of isoenzymes and should be regarded as preliminary.

§PDE III/IV characterization incomplete.

¶Preliminary report/classification based on selective inhibitor potency.

[a]Reeves, M. L. *et al.* (1987) *Biochem. J.* 241, 535–541; [b]Hidaka, H. *et al.* (1977) *Biochim. Biophys. Acta* 484, 398–407; [c]Masuoka, H. *et al.* (1990 *J. Cardiovasc. Pharmacol.* 15, 302–307; [d]Kithas, P. A. *et al.* (1989) *J. Mol. Cell. Cardiol.* 21, 507–517; [e]Shahid, M. and Nicholson, C. D. *Naunyn Schmiedeberg's Arch. Pharmacol.* (in press); [f]Shahid, M. *et al.* (1990) *J. Pharm. Pharmacol.* 42, 283–284; [g]Kithas, P. A. *et al.* (1988) *Circ. Res.* 62 782–789; [h]Klockow, M. and Jonas, R. (1989) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 339, R53; [i]Weishaar, R. E. *et al.* (1987) *J. Mol. Cell. Cardiol.* 19, 1025–1036; [j]Komas, N. *et al.* (1989) *J. Cardiovasc. Pharmacol.* 14, 213–220; [k]Kauffmann, R. F. *et al.* (1986) *Mol. Pharmacol.* 30, 609–615

tiation of the cAMP-elevating actions of β-adrenoceptor agonists. Kinetic analysis clearly demonstrates that despite only representing a small proportion of total hydrolytic activity, PDE IV plays a crucial role in regulating the rate of cAMP hydrolysis in this tissue (Fig. 2).

Studies of the relative roles of different isoenzymes in cyclic nucleotide regulation, and the determination of the importance of receptor-mediated control of hydrolytic activity, have recently been simplified by several advances in methodology (see Box).

Selective inhibitors of phosphodiesterase isoenzymes

Since the initial discovery of the ability of methylxanthines to inhibit phosphodiesterase activity, considerable advances have been made in increasing our understanding of the structural requirements of phosphodiesterase inhibitors. The use of theophylline and caffeine as tools to examine the functional significance of phosphodiesterase inhibition is limited by their other pharmacological activities, including adenosine receptor antagonism[15] and an ability to inhibit the guanine nucleotide-binding protein G_i (Ref. 16). Alkylxanthines have now been synthesized that, in contrast to theophylline, caffeine and IBMX, selectively inhibit particular phosphodiesterase isoenzymes (e.g. denbufylline and 1-methyl-2-isobutyl-8-methoxyethyl xanthine; Table I), but a complete elucidation of their pharmacological profiles is required before they can confidently be used to examine the functional aspects of isoenzyme selective inhibition.

The most extensively examined phosphodiesterase isoenzyme is PDE III. Inhibitors of this isoenzyme have some structural similarity to the natural substrate, cAMP. Computational medicinal chemistry has allowed a template for generating further PDE III inhibitors to be proposed[17]. Although many selective inhibitors for PDE III have been developed, their absolute specificity varies (Table I). Many experiments in which the functional activity of PDE III inhibitors has been examined have used nonselective concentrations; many of these

Tissue	Soluble PDE: Isoenzyme	Species	Methods*	% Activity	Particulate PDE: Isoenzyme	Species	Methods*	Organelle†	% Activity
Trachea	I, II, III/IV[§], V	dog[ee]	A/D	80–90[ee]	I/III/IV	dog[ee]	D‡	–	10–20[ee]
		bovine[ff]	A	>90[ff]	I/IV	bovine[ff]	A	–	0.5–2[ff]
	I, II, III/IV[§], V	human[gg]	A	–					
Myometrium	I, IV, V	human[hh]	C	–					
Adipose	I, II	bovine[ii]	B	–	III	bovine[ii]	B	–	80[ii]
	I, II, III	rat[ii]	D	30[jj]	III	rat[jj,kk]	D	ER/PM	70[jj,kk]
					III	rat[ll]	D	ER	–
					II	rat[ll]	D	nucleus/mito	–
Kidney	I, II, III/IV[§]	human[b]	A	40–75[b]	–	human[b]	A	–	25–50[b]
					IV	human[c]	D	–	–
	I, II, IV	rat[mm]	D	80–90[mm]	III	rat[mm]	D	–	–
Platelets	I, II, III, V	human[nn]	A	85[oo]	III	bovine and	A	–	15[oo]
	II, III, V	human[pp]	A	–	–	human[oo]		–	–
Lung	I, III/IV[§], V	human[qq]	A	89–90[qq]	–	human[b]	A	–	25–40[b]
	I, II, III/IV[§]	bovine[rr]	A	–					
Bronchi	II, III/IV[§], V	human[ss]	A	78–86[ss]					
Endothelium	II, IV	bovine[tt]	B	80[tt]	II	bovine[tt]	B	–	–
Neutrophils					IV	human[uu]	D	–	80[uu]
Monocytes	IV[¶]	human[vv]							
Mast cells	III/IV[§], V	rat[ww]	C	80[ww]	–	rat[ww]	C	–	–

[l]Nicholson, C. D. *et al.* (1989) *Br. J. Pharmacol.* 97, 889–897; [m]Ahluwalia, G. *et al.* (1984) *Int. J. Biochem.* 16, 483–488; [n]Komas, N. *et al.* (1990) *J. Mol Cell. Cardiol.* 22 (Suppl. IV), 532; [o]Weishaar, R. E. *et al.* (1987) *Circ. Res.* 61, 539–547; [p]Timouyasse, L. *et al.* (1989) *Biochem. Int.* 19, 287–299 [q]Simmons, M. A. and Hartzell, H. C. (1988) *Mol. Pharmacol.* 33, 664–671; [r]Lavan, B. E. *et al.* (1989) *Biochem. Pharmacol.* 38, 4123–4136; [s]Pyne, N. J *et al.* (1987) *Biochem. J.* 242, 33–42; [t]Yamamoto, T. *et al.* (1984) *Biochemistry* 23, 670–675; [u]Pyne, N. J. *et al.* (1986) *Biochem. J.* 234, 325–334 [v]Strada, S. J. *et al.* (1984) in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Vol. 16) (Strada, S. J. and Thompson, W. J., eds) p. 13, Raven Press; [w]Wilke, R. (1990) *Eur. J. Pharmacol.* 183, 1367; [x]Whalin, M. E. *et al.* (1988) *Biochim. Biophys. Acta* 972, 79–94; [y]Egrie, J. C. *et al.* (1977) *J. Neurochem.* 28, 1207–1213; [z]Ahn, H. S. *et al.* (1989) *Biochem. Pharmacol.* 38, 3331–3339; [aa]O'Connor, B. and Silver, P. J. (1990) *Drug Dev. Res.* 19, 435–442; [bb]Lugnier, C. *et al.* (1986) *Biochem. Pharmacol.* 35, 1743–1751; [cc]Prigent, A. F. *et al.* (1988) *Biochem. Pharmacol.* 37, 3671–3681; [dd]Shahid, M. (unpublished); [ee]Torphy, T. J. and Cieslinski, L. B. (1990) *Mol. Pharmacol.* 37, 206–214; [ff]Shahid, M. *et al. Br. J. Pharmacol.* (in press); [gg]Cielinski, L. B. *et al.* (1988) *FASEB, J.* 2, 4474; [hh]Leroy, M. J. *et al.* (1989) *Biochem. Pharmacol.* 38, 9–15; [ii]Degerman, E. *et al.* (1988) *Second Messengers Phosphoproteins* 12, 171–182; [jj]Manganiello, V. C. *et al.* (1988) *Methods Enzymol.* 159, 504–520; [kk]Boyes, S. and Loten, E. G. (1989) *Biochem. Biophys. Res. Commun.* 162, 814–820; [ll]Anderson, N. G. *et al.* (1989) *Biochem. J.* 262, 867–872; [mm]Hoey, M. and Houslay, M. D. (1990) *Biochem. Pharmacol.* 40, 193–202; [nn]Simpson, A. W. M. *et al.* (1988) *Biochem. Pharmacol.* 37, 2315–2320; [oo]MacPhee, C. H. *et al.* (1986) *Proc. Natl Acad. Sci. USA* 83, 6660–6663; [pp]Hidaka, H. and Endo, T. (1984) in *Advances in Cyclic Nucleotide Protein Phosphorylation Research* (Vol 16) (Strada, S. J. and Thompson, W. J., eds), pp. 245–249, Raven Press; [qq]Bergstrand, H. and Lundquist, B. (1976) *Biochemistry* 15, 1727–1734; [rr]Beavo, J. A. *et al.* (1982) *Mol. Cell. Endocrinol.* 28, 387–410; [ss]Bergstrand, H. and Lundquist, B. (1978) *Mol. Cell. Biochem.* 21, 9–15; [tt]Lugnier, C. and Schini, V. B. (1990) *Biochem. Pharmacol.* 40, 699–707; [uu]Wright, C. D. *et al.* (1990) *Biochem. Pharmacol.* 40, 699–707; [vv]White, J. R. *et al.* (1990) *FASEB J.* 4, 1710; [ww]Bergstrand, H. *et al.* (1978) *Mol. Pharmacol.* 14, 848–855.

compounds, like other isoenzyme-selective inhibitors, have additional activities at higher concentrations that may also contribute to their pharmacological effects.

Compounds with some selectivity for PDE I, PDE IV and PDE V have also been reported (Table I). A systematic study of alkylxanthines has revealed the structural requirements around the xanthine nucleus for compounds with apparent selectivity for PDE I[18]. However, many of these compounds are 8-substituted xanthines and thus may also have affinity for adenosine receptors[19]. Additionally, recent studies have demonstrated that PDE V often co-elutes with PDE I from chromatographic columns[1]. It is possible therefore that, in many studies in which PDE I activity has reportedly been assayed, a mixture of PDE I and PDE V was actually examined. Consequently, the absolute specificity of PDE I selective inhibitors such as 1-methyl-2-isobutyl-8-methoxyethyl xanthine requires confirmation. Structure–activity relationships for PDE IV and PDE V inhibitors have yet to be reported while selective inhibitors for PDE II have not been identified.

Modulation of tissue function by isoenzyme inhibitors

Functional effects of selective inhibitors have also been used to assess the relative importance of different isoenzymes on cyclic nucleotide hydrolysis. However, many of these experiments have been performed using isolated tissue preparations where basal turnover rates of cyclic nucleotides are low and high concentrations of phosphodiesterase inhibitors are consequently necessary to elevate cyclic nucleotide levels and to produce a change in tissue function. *In vivo*, cyclic nucleotide turnover rates are enhanced and phosphodiesterase inhibitors consequently have much greater effects on tissue cyclic nucleotide levels.

PDE III and PDE IV inhibitors

Although both PDE III and PDE IV have high activity and affinity for cAMP, and hence may be expected to function over approximately the same range of substrate concentrations, their effects on

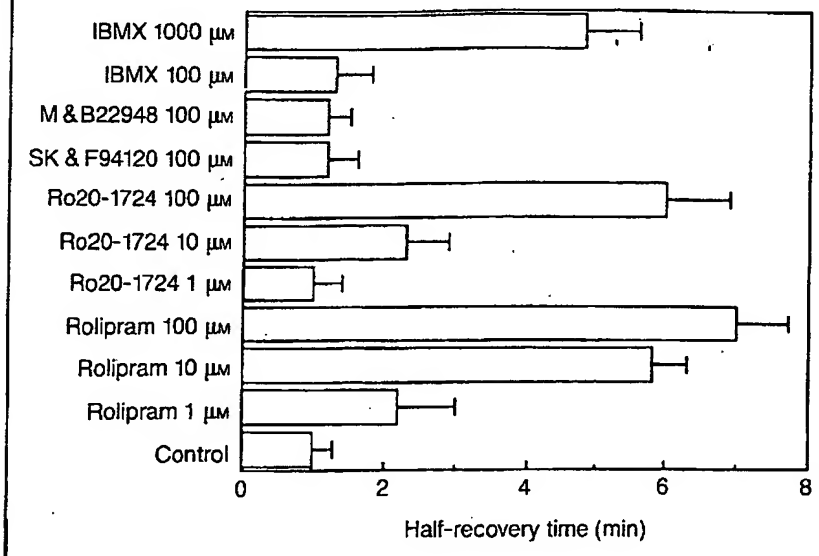

Fig. 2. The effect of PDE inhibitors on the rate of cAMP hydrolysis in rat cerebral cortex slices. Experiments were performed as described in Ref. 14. Cerebral cortex slices were incubated for 30 min with the PDE inhibitor before addition of isoprenaline (10 μM); 20 min later timolol (20 μM) was added to displace isoprenaline from all β-adrenoceptors. The rate of cAMP hydrolysis was then followed with time. Selective inhibitors of PDE IV (Ro20–1724 and rolipram), but not inhibitors of the PDE V (M&B22948, zaprinast) or PDE III (SK&F94120), effectively slowed cAMP hydrolysis rates. (Modified from Ref. 12.)

tissue function differ. For example, inhibitors of PDE III, but not PDE IV, are positive inotropic agents in most mammals[10]. It has been suggested that PDE III inhibitors act by inhibiting a particulate pool of PDE III[10,12]. However, experimental evidence suggests that inotropic responses may not be solely due to inhibition of particulate phosphodiesterase[20]. Rather it may be due to inhibition of a sufficient proportion of phosphodiesterase isoenzymes capable of hydrolysing cAMP. In rat cardiac muscle, the particulate phosphodiesterase is PDE IV[21] and neither selective inhibitors of PDE III nor PDE IV increases force of contraction when given alone, but they do increase contractility when administered in combination (Fig. 3). In rabbit heart muscle, in which a significant proportion of PDE III is membrane bound, PDE III inhibitors do increase contractility when administered alone[20]. However, simultaneous inhibition of PDE IV potentiates the inotropic activity of PDE III inhibitors[20]. Perhaps inhibition of a sufficient proportion of total phosphodiesterase activity in a relevant intracellular locale is generally important for the modulation of cell function. This proposal is supported by work with guinea-pig trachea. In this tissue, combined PDE III and PDE IV inhibition produces more effective inhibition of total cAMP hydrolytic activity and greater tracheal relaxation than selective inhibition of either isoenzyme alone[7]. Species differences in the ability of PDE III and PDE IV inhibitors to modulate tissue function are not confined to cardiac muscle. In bovine tracheal preparations PDE IV, but not PDE III, selective inhibitors are effective relaxants[8], while in guinea-pig trachealis, both PDE III and PDE IV inhibitors attenuate contractions[7]. This is consistent with the presence of appreciable PDE III and PDE IV activity in guinea-pig trachealis[7] and the lack of marked PDE III activity in bovine trachealis[8].

Selective inhibitors of PDE III elicit other pharmacological effects, including relaxation of vascular smooth muscle[9], inhibition of platelet aggregation[22] and stimulation of lipolysis[23]. Despite the fact that PDE IV is present in smooth muscle cells (Table II), selective inhibitors of PDE IV have not been demonstrated to have marked vasodilator potential. Additionally, PDE IV inhibitors are weak platelet aggregation inhibitors[24] and lipolytic agents[23]. Although PDE III is often considered as a high-affinity or low K_m cAMP phosphodiesterase, unlike PDE IV it also hydrolyses cGMP with high affinity. This action may be important in the regulation of vascular smooth muscle tone[9]. Hence, the functional effects of PDE III inhibition, but not PDE IV inhibition, may be mediated via elevation of both cellular cGMP and cAMP levels.

It is established that phosphodiesterase inhibitors attenuate the release of inflammatory mediators from human lung tissue[25]. Preliminary data indicate that PDE IV predominates in inflammatory cells; selective inhibitors of this isoenzyme, in contrast to PDE III inhibitors, effectively inhibit the release of inflammatory mediators[26]. This suggests that PDE IV inhibitors have not only bronchodilating activity, but also anti-inflammatory activity, an important consideration in asthma (see below).

Inhibitors of PDE IV produce centrally mediated and characteristic alterations in animal behaviour[27] and improve the performance of animals in certain paradigms of cognition[28]. The PDE IV selective inhibitor denbufylline enhances the excitability of hippocampal neurons[29], providing a functional basis for this cognition-enhancing activity. In the hippocampus, noradrenaline produces a β-adrenoceptor-mediated enhancement of excitatory responses to glutamate[30], a neurotransmitter intimately involved in learning and memory. The mechanism of action of PDE IV inhibitors on neuronal excitability and cognition may thus involve cAMP-mediated enhancement of responses to glutamate. The lack of an identifiable presence of PDE III in cerebral cortical tissue[31] and the absence of reports indicating central activity suggests that inhibitors of this enzyme do not modulate brain function.

Other functional effects selectively associated with PDE IV inhibitors include increased gastric acid secretion[32], enhanced contractility of ischaemic fast twitch skeletal muscle[33] and relaxation of the myometrium[34]. PDE IV is also believed to play a regulatory role in diuresis in the kidney[4] and in glycogenolysis in the liver[3].

New developments in methodology

It has been appreciated for at least 20 years that cellular phosphodiesterase activities are subject to direct or indirect receptor-mediated regulation[1]. However, until recently few studies have unambiguously defined the phosphodiesterase isoenzyme affected by external control and the consequences for regulation of cyclic nucleotide levels. There is evidence for the hormonal regulation of membrane-bound phosphodiesterases through guanine nucleotide-binding proteins[2,3] or cAMP-and Ca^{2+}/calmodulin-dependent protein kinases to provide rapid feedback controls[4,5]. A slower cAMP-mediated control involving altered production of phosphodiesterase mRNA transcripts has also been reported[6]. The further investigation of such control mechanisms and the role of individual isoenzymes in controlling cyclic nucleotide hydrolysis is now more feasible because of a number of methodological developments:

- Determination of cAMP turnover using a kinetic dual-labelling technique[7].
- Use of cell-permeant, hydrolysable cyclic nucleotide analogues (e.g. 8-*p*-chlorophenylthio-cAMP) to study hormone-stimulated changes in phosphodiesterase isoenzymic activities[8].
- Use of $H_2{}^{18}O$ labelling of the cellular nucleotide pool to study cyclic nucleotide turnover and compartmentation[9].
- Analysis of the localization of phosphodiesterase isoenzymes can be performed using radiolabelled isoenzyme selective inhibitors. [^{3}H]Rolipram specifically binds to cerebral cortical membrane-associated and cytosolic sites[10] which may be located on PDE IV. The affinity of rolipram for these binding sites is high in comparison with its ability to inhibit crude PDE IV. This discrepancy has yet to be fully explained, however, rolipram may selectively bind with high affinity to a particular PDE isoform. [^{3}H]LY186126 has been developed as a radiolabel for PDE III and the compound has been demonstrated to bind with high affinity to a site on cardiac sarcoplasmic reticulum vesicles[11].
- cDNA clones representing individual phosphodiesterase isoenzymes are now becoming available and the homology between animal and human clones is being investigated[12].

A fascinating aspect of these developments is the highlighting of the apparent coordinate hormonal activation of cAMP synthesis and hydrolysis that can occur in some systems[9,13]. There is evidence that dramatic changes in the ATP–cAMP–5'AMP flux can occur with little or no change in cAMP accumulation[9]. Whether this represents an efficient feedback loop eliciting a transient change in second messenger concentration despite ongoing receptor stimulation, or a unique signalling system dependent on changes in pathway flux rather than the amplitude of change in second messenger concentration, has yet to be clarified. It may however explain the long-standing puzzle of why changes in tissue function can be produced either by phosphodiesterase inhibitors or by agonists at extracellular receptors linked to adenylyl cyclase with little change in cellular cAMP levels.

References

1 Loten, E. G. and Sneyd, J. G. T. (1970) *Biochem. J.* 120, 187–193
2 Manganiello, V. C. (1987) *J. Mol. Cell. Cardiol.* 19, 1037–1040
3 de Mazancourt, P. and Guidicelli, Y. (1988) *Methods Enzymol.* 159, 766–772
4 Anderson, N. G., Kilgour, E. and Houslay, M. D. (1989) *Biochem. J.* 262, 867–872
5 Sharma, R. K. and Wang, J. H. (1986) *Biochem. Cell. Biol.* 64, 1072–1080
6 Swinnen, J. V., Joseph, D. R. and Conti, M. (1989) *Proc. Natl. Acad. Sci. USA* 86, 8197–8201
7 Barber, R. and Butcher, R. W. (1988) *Methods Enzymol.* 159, 50–60
8 Gettys, T. W., Blackmore, P. F. and Corbin, J. D. (1988) *Am. J. Physiol.* 254, E449–E453
9 Deeg, M. A., Graeff, R. M., Walseth, T. F. and Goldberg, N. D. (1988) *Proc. Natl Acad. Sci. USA* 85, 7867–7871
10 Schnieder, H. H., Schmiechen, R., Brezinski, M. and Seidler, J. (1986) *Eur. J. Pharmacol.* 127, 105–115
11 Kauffman, R. F., Utterback, B. G. and Robertson, D. W. (1989) *Circ. Res.* 64, 1037–1040
12 Livi, G. P. *et al.* (1990) *Mol. Cell. Biol.* 10, 2678–2686
13 MacPhee, C. H., Reifsnyder, D. H., Moore, T. A., Lerea, K. M. and Beavo, J. A. (1988) *J. Biol. Chem.* 263, 10353–10358

PDE I, PDE II, PDE V inhibitors

Much less effort has been expended in synthesizing selective inhibitors of PDE I, PDE II and PDE V and hence less is known about the functional consequences of selective inhibition of these isoenzymes. However, it is recognized that inhibitors of PDE V are potent inhibitors of platelet aggregation, but are weak cardiac inotropic agents and poor vascular relaxants[22]. By contrast, selective inhibitors of PDE I are effective vascular relaxants, but poor platelet anti-aggregatory agents[22]. The difference in activity profile may reflect the absolute or relative differences in importance of PDE I and PDE V on cGMP hydrolysis in platelets and vascular smooth muscle. Inhibitors of PDE I also enhance cognition in animals[28], indicating that such compounds may modulate some aspects of cerebral cortical function. Zaprinast, which inhibits PDE V at low concentrations and PDE I at higher concentrations, is both a bronchodilator and an inhibitor of histamine release from mast cells[35]. It is not known whether PDE I or PDE V is responsible for these effects. However, given the role of mast cells in inflammatory conditions, this activity may indicate anti-inflammatory activity for this type of compound. Selective inhibitors of PDE II have not yet been identified.

Clinical potential

The ubiquitous nature of cAMP and cGMP and their role in controlling tissue function makes phosphodiesterase inhibition an obvious target for drug development. However, in many cases selective inhibitors have been synthesized serendipitously within disease-oriented programmes, rather than as a result of targeted chemical synthesis. Nevertheless, phosphodiesterase isoenzyme in-

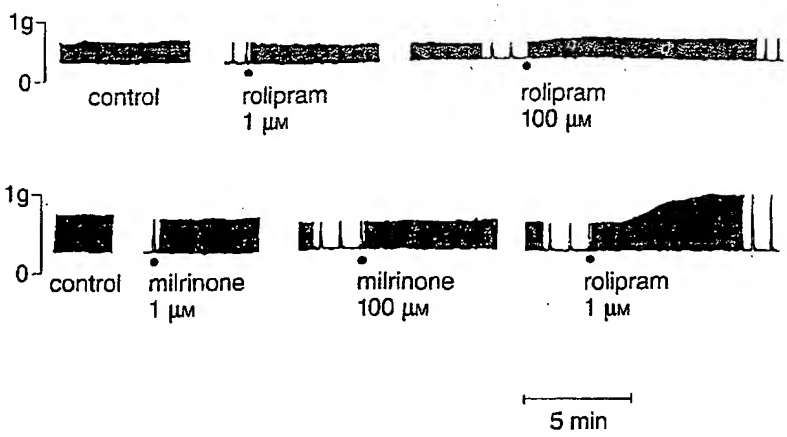

Fig. 3. The effect of rolipram and milrinone on the tension developed by electrically stimulated papillary muscles removed from rat right cardiac ventricles. Complete cumulative concentration–response curves were constructed for both rolipram (1–100 μM) and milrinone (1–100 μM), the mechanograms were obtained from two different preparations and the results shown are those obtained with the lowest and highest concentration only. Following the completion of the milrinone concentration–response curve, rolipram was added to the organ bath without washing. Neither rolipram nor milrinone increased contractility when administered alone; however, in the presence of milrinone, rolipram effectively increased developed tension. This indicates that dual inhibition of PDEs IV and V more effectively increases cardiac contractility than selective inhibition of either isoenzymes. (From Ref. 20.)

hibitors are now being considered for development in several clinical disorders.

Congestive heart failure

The balanced therapy of heart failure involves supporting cardiac contractility, reducing cardiac load and improving blood flow to organs such as the kidney and skeletal muscles. Because of their vasodilator and inotropic activities, PDE III inhibitors are undergoing evaluation as drugs for the treatment of congestive heart failure[36]. Although useful for the acute treatment of the disorder, phosphodiesterase inhibitors have arrhythmogenic potential and it remains to be proven that they reduce mortality when given chronically. Dual inhibition of both PDE III and PDE IV produces greater inotropic activity than selective inhibition of either isoenzyme. Thus, dual inhibitors of both low K_m cAMP phosphodiesterase isoenzymes may be more effective cardiotonics than selective PDE III inhibitors. However, compounds with ancillary properties such as Ca^{2+} sensitization (e.g. pimobendan and Org30029; see Table I) and antiarrhythmic activity may prove even more useful agents for the treatment of heart failure. A 12-week, double-blind, placebo-controlled study with OPC8212, a PDE III inhibitor that also slows the rate of closure of Na^+ channels[37], has suggested that this agent may improve prognosis in patients with mild heart failure[38]. Of 38 placebo-treated patients, two died and another six were withdrawn because of deterioration of heart failure. Of 45 OPC8212-treated patients, only one was withdrawn because of increased congestive symptoms. After 12 weeks of treatment, the OPC8212-treated patients had increased ejection fractions and reduced systolic blood pressure, heart rate was not increased and the patients had an increased sense of well-being. The clarification of whether PDE III inhibitors with ancillary properties are therapeutically more useful drugs than selective PDE III inhibitors is an important area of future clinical research.

Asthma

Asthma is an inflammatory condition characterized by invasion of the airways by inflammatory cells, bronchoconstriction, microvascular leakage, mucus hypersecretion and bronchial hyperresponsiveness[39]. Drug therapy can be aimed either at inhibiting the underlying inflammatory reaction or at attenuating the resultant narrowing of the airways. Theophylline has long been widely used for the therapy of asthma. Although the precise mechanism of action of this agent remains to be determined, evidence has again been presented indicating that phosphodiesterase inhibition may be significant at clinically relevant concentrations, and hence may be responsible for the anti-inflammatory[40] as well as the bronchodilator effects of theophylline[41]. Their ability to relax airways smooth muscle suggests that inhibitors of PDE III, PDE IV and possibly both PDE I and PDE V may be useful in the acute relief of asthmatic attacks. The anti-inflammatory activity of PDE IV inhibitors makes these agents especially attractive as candidates for use in asthma prophylaxis, while the ability of inhibitors of PDE V to attenuate histamine release from mast cells may also be advantageous. Platelets also play a role in asthmatic attacks[39] and because PDE III inhibitors, but not PDE IV inhibitors, inhibit platelet aggregation, dual inhibitors of PDE III and PDE IV may prove to be therapeutically more powerful than selective inhibitors of either isoenzyme. However, the possibility of unwanted side-effects may also be greater with a dual PDE III/IV inhibitor. In particular, the cardiovascular effects of PDE III and PDE III/IV inhibitors may be a barrier to the systemic use of such agents in asthma therapy.

Antagonists targeted against single mediators are unlikely to be universally effective drugs in the treatment of asthma, as a diverse range of cell types and chemical mediators are involved in the pathology of the disease. Agents that functionally antagonize the bronchoconstriction, airways hyperreactivity and inflammatory processes at sites distal to extracellular receptors may be the most effective therapeutic agents. Hence, PDE IV, dual PDE III/IV and PDE V inhibitors are potentially interesting agents worthy of clinical evaluation in asthmatics. Many of the side-effects of theophylline therapy such as nausea and headache may be due to PDE inhibition. Whether these can be avoided in isoenzyme selective inhibitors remains to be explored.

Central disorders

Inhibitors of PDE IV have mood-elevating properties. For example, rolipram has been reported to

possess anti-depressant properties[42]. In addition the PDE IV inhibitor, denbufylline, is being evaluated for the treatment of senile dementia[28]. Like asthma, primary senile dementia is of multifactorial aetiology which complicates rational therapy. Symptoms can either be caused by vascular occlusion in multi-infarct dementia, or by neuronal degeneration in senile dementia of the Alzheimer type. In the latter case, the symptoms are unlikely to be due to selective degeneration of a particular neuronal system[43]. Although cholinergic and glutamatergic degeneration may be responsible for the cognitive decline, dysfunction in noradrenergic or 5-HT-containing neurons contributes to the non-cognitive changes in behaviour[43]. Consequently, PDE IV and possibly PDE I inhibitors, which may elevate both mood and cognition, could prove to be useful agents for the treatment of milder forms or the early stages of dementia.

Arterial thrombosis and hypertension

Inhibitors of PDE V have potential for the treatment of arterial thrombosis. Ideally, such anti-thrombotics should inhibit platelet aggregation without causing relaxation of vascular smooth muscle. The latter activity may cause redistribution of blood away from already underperfused tissue in ischaemic vascular conditions. Inhibitors of both PDE V and PDE III inhibit platelet aggregation[22], but unlike inhibitors of PDE III, compounds inhibiting PDE V are relatively poor vasodilators[22]. Because of their vasodilating activity, inhibitors of PDE III and PDE I may have potential in the management of hypertension. However, selectivity of action may be a major barrier to the use of these agents in the therapy of this disorder and an advantage of these agents in comparison to the presently utilized vasodilators is not yet apparent.

Finally, other possible clinical uses of phosphodiesterase inhibitors include the management of intermittent claudication (alkylxanthines are currently used in this disorder), preterm labour (phosphodiesterase inhibitors block uterine contraction) and psoriasis. Cyclic nucleotide metabolism is disturbed in psoriatic skin and phosphodiesterase inhibitors have shown some clinical efficacy in psoriasis[44].

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References

1 Beavo, J. A. (1988) in *Advances in Cyclic Nucleotide and Protein Phosphoprotein Research* (Vol. 22) (Greengard, P. and Robison, G. A., eds), pp. 1–38, Raven Press
2 Beavo, J. A. and Reifsnyder, D. H. (1990) *Trends Pharmacol. Sci.* 11, 150–155
3 Lavan, B. E., Lakey, T. and Houslay, M. D. (1989) *Biochem. Pharmacol.* 38, 4123–4136
4 Hoey, M. and Houslay, M. D. (1990) *Biochem. Pharmacol.* 40, 123–202
5 Kukovetz, W. R., Holzman, S., Wurm, A. and Poch, G. (1979) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 310, 129–136
6 Lorenz, K. and Wells, J. N. (1983) *Mol. Pharmacol.* 23, 424–430
7 Harris, A. L. *et al.* (1986) *J. Pharmacol. Exp. Ther.* 251, 198–206
8 Nicholson, C. D., Shahid, M. van Amsterdam, R. G. M. and Zaagsma, J. (1990) *Eur. J. Pharmacol.* 183, 1097–1098
9 Kauffman, R. F., Schenk, K. W., Utterbach, B. G., Crowe, V. G. and Cohen, M. L. (1987) *J. Pharmacol. Exp. Ther.* 242, 864–872
10 Weishaar, R. E., Kobylarz-Singer, D. C., Steffen, N. P. and Kaplan, H. R. (1987) *Circ. Res.* 61, 539–547
11 Silver, P. J. (1989) *Am. J. Cardiol.* 63, 3A–8A
12 Kauffman, R. F., Crowe, V. G., Utterback, B. G. and Robertson, D. W. (1986) *Mol. Pharmacol.* 30, 609–616
13 England, P. J. and Shahid, M. (1987) *Biochem. J.* 246, 687–695
14 Challiss, R. A. J. and Nicholson, C. D. (1990) *Br. J. Pharmacol.* 100, 47–53
15 Fredholm, B. B. (1980) *Trends Pharmacol. Sci.* 1, 129–132
16 Parsons, W. D., Ramkumar, V. and Stiles, G. L. (1988) *Mol. Pharmacol.* 34, 37–41
17 Moos, W. H. *et al.* (1987) *J. Med. Chem.* 30, 1963–1972
18 Wells, J. N., Garst, J. E. and Kramer, G. L. (1981) *J. Med. Chem.* 24, 956–958
19 Hamilton, H. W. *et al.* (1985) *J. Med. Chem.* 28, 1071–1079
20 Shahid, M. and Nicholson, C. D. *Naunyn-Schmiedeberg's Arch. Pharmacol.* (in press)
21 Shahid, M., Wilson, M., Nicholson, C. D. and Marshall, R. J. (1990) *J. Pharm. Pharmacol.* 42, 283–284
22 Hidaka, H. and Endo, T. (1984) in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Vol. 16) (Strada, S. J. and Thompson, W. J., eds), pp. 245–259, Raven Press
23 Manganiello, V. C. (1987) *J. Mol. Cell. Cardiol.* 19, 1037–1040
24 Simpson, A. W. M., Reeves, M. L. and Rink, T. J. (1988) *Biochem. Pharmacol.* 37, 2315–2320
25 Kaliner, M. and Austen, K. F. (1978) *Biochem. Pharmacol.* 23, 763–771
26 Dent, G., Giembycz, M. A. and Barnes, P. J. (1990) *Br. J. Pharmacol.* 99, 163P
27 Wachtel, H. (1982) *Psychopharmacology* 77, 309–316
28 Nicholson, C. D. (1990) *Psychopharmacology* 101, 147–159
29 Sutor, B., Alzheimer, C., Ameri, A. and ten Bruggencate, G. (1990) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 342, 349–356
30 Nicoll, R. A., Madison, D. V. and Lancaster, B. (1987) in *Psychopharmacology the Third Generation of Progress* (Meltzer, H. Y., ed.), pp. 105–112, Raven Press
31 Nicholson, C. D., Jackman, S. A. and Wilke, R. (1989) *Br. J. Pharmacol.* 97, 889–897
32 Black, E. W., Strada, S. K. and Thompson, W. J. (1988) *J. Pharmacol. Methods* 20, 57–78
33 Angersbach, D. and Ochlich, P. (1984) *Arzneim.-Forsch. Drug Res.* 34, 1724–1728
34 Leroy, M. J., Cedrin, I., Breullier, M., Giovagrandi, Y. and Ferre, Y. (1989) *Biochem. Pharmacol.* 38, 9–15
35 Frossard, N. Landry, Y. Pauli, G. and Ruchstuhl, M. (1981) *Br. J. Pharmacol.* 73, 933–938
36 Wetzel, B. and Hauel, N. (1989) *Trends Pharmacol. Sci.* 9, 166–170
37 Lathrop, D. A. and Schwartz, A. (1985) *Eur. J. Pharmacol.* 117, 391–397
38 OPC 8212 Multicenter Research Group (1990) *Cardiovasc. Drugs Ther.* 4, 419–426
39 Barnes, P. J., Chung, K. F. and Page, C. (1988) *Pharmacol. Rev.* 40, 49–84
40 Krzanowski, N. and Polson, J. B. (1988) *J. Allergy. Clin. Immunol.* 82, 143–145
41 Nielson, C. P., Crowley, J. J., Cusak, B. and Vestal, R. E. (1986) *J. Allergy Clin. Immunol.* 78, 660–667
42 Eckmann, F., Fichte, K., Meya, U. and Sastre-Y-Hernandez, M. (1988) *Curr. Ther. Res.* 43, 291–295
43 Palmer, A. M. and Gershon, S. (1990) *FASEB J.* 4, 2745–2752
44 Vorhees, J. J. and Marcelo, C. (1977) in *Psoriasis* (Forbes, E. M. and Cox, A. J., eds), pp. 91–99, Yorke Medical Books

EMD54622: (5-(1-(3,4-dimethoxybenzoyl)-4,4-di-methyl-1,2,3,4-tetrahydrocinnolin-6-yl)-6-methyl-3,6-dihydro-1,3,4-thiadiazin-2-one)
ICI118233: *N*-[4-(1,6-dihydro-6-oxo-3-pyridazinyl)phenyl]-*N*-methyl-urea
ICI63197: 2-amino-6-methyl-4-propyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-5(4*H*)-one
LY186126: 1,3-dihydro-1,3,3-trimethyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one
MY5445: *N*-(3-chlorophenyl)-4-phenyl-1-phthalazinamine
M&B22948: 1,4-dihydro-5-(2-propoxyphenyl)-7*H*-1,2,3-triazolo[4,5-d]pyrimidin-7-one
OPC8212: 1-(3,4-dimethoxybenzoyl)-4-(1,2,3,4-tetrahydro-2-oxo-6-quinolinyl)-piperazine
Org30029: *N*-hydroxy-5,6-dimethoxybenzo[*b*]thiophene-2-carboximidamine hydrochloride
RS82856: *N*-cyclohexyl-*N*-methyl-4-[(1,2,3,5-tetrahydro-2-oxoimidazo[2,1-*b*]-quinazolin-7-yl]oxy]-butanamide, sulphate (1:1)
Ro20-1724: 4-[(3-butyoxy-4-methoxyphenyl)methyl]-2-imidazolidinone
SK&F94120: *N*-[4-(4,5-dihydro-5-oxopyrazinyl)phenyl]-acetamide